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Getting under the skin of p63

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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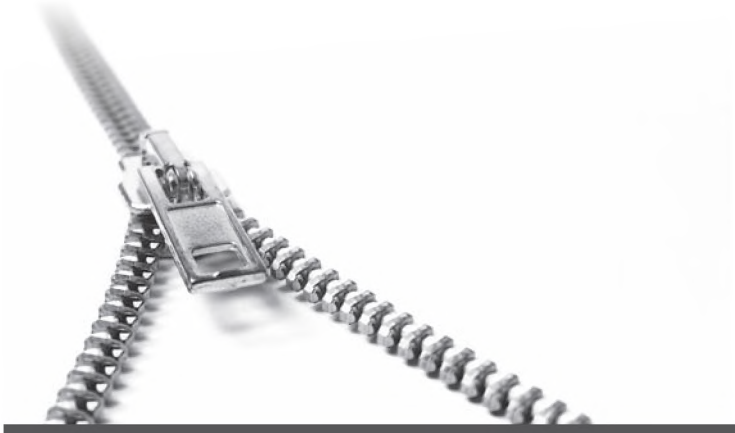

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Getting under the skin of p63

An academic essay in Medical Sciences

DOCTORAL THESIS

To obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the
Rector Magnificus prof. dr. S.C.J.J. Kortmann,
according to the
decision of the council of deans
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by
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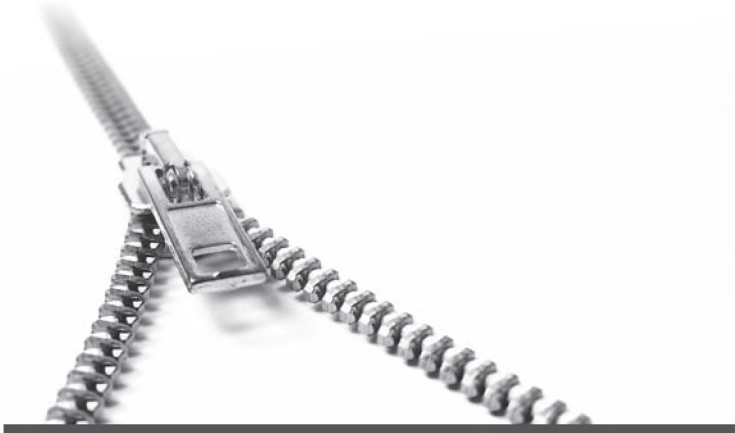

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Abbreviations

A	Adenine (DNA/RNA)
A	Alanine (Ala) (amino acid)
ACTB	Beta-actin
ADULT	Acro-dermato-ungual-lacrimal-tooth
AEC	Ankyloblepharon ectodermal defects cleft lip/palate
AER	Apical ectodermal ridge
Bp	Basepair
BMP	Bone morphogenetic protein
C	Cytosine (DNA/RNA)
C	Cysteine (Cys) (amino acid)
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immuno-precipitation
ChIP-on-chip	Chromatin immuno-precipitation on DNA array
CLDN	Claudin
CLP	Cleft lip/palate
CMV	Cytomegalovirus
CX	Connexin
D	Aspartic acid (Asp)
DNA	Deoxyribonucleic acid
DLX	Distal-less homeobox gene
E	Glutamic acid (Glu)
ED	Ectodermal dysplasia
EEC	Ectrodactyly, ectodermal dysplasia and cleft lip/palate
EGF	Epidermal growth factor
F	Phenylalanine (Phe)
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
fs	Frameshift
G	Guanine (DNA/RNA)
G	Glycine (Gly) (amino acid)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase protein
hARP	Human Acidic ribosomal protein
H	Histidine (His)
I	Isoleucine (Ile)
ISO	Isomerization (domain)
K	Lysine (Lys)
Kb	Kilobase
kDa	KiloDalton
KGM	Keratinocyte growth medium
KO	Knock-out
KRT	Keratin
L	Leucine (Leu)
LADD	Lacrimo-auriculo-dento-digital
LMS	Limb mammary syndrome
M	Methionine
mRNA	Messenger ribonucleic acid

N	Asparagine (Asn)
NFkB	Nuclear factor k beta
NMD	Nonsense-mediated RNA decay
NSCL	Non-syndromic cleft lip/palate
nt	Nucleotide
OD	Oligomerization
P	Proline (Pro)
p.	Protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTC	Premature termination codon
Q	Glutamine (Gln)
qPCR	Quantitative polymerase chain reaction
R	Arginine (Arg)
RHS	Rapp-Hodgkin syndrome
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Serine (Ser)
SAM	Sterile alpha motif (domain)
SCC	Squamous cell carcinoma
SHFM	Split hand/foot malformation
SHH	Sonic hedgehog homolog
SNP	Single nucleotide polymorphism
T	Thymine (DNA/RNA)
T	Threonine (Thr) (amino acid)
TA	Transactivation (domain)
TA2	Second transactivation (domain)
TGF	Transforming growth factor
TI	Transactivation inhibitory (domain)
TNF	Tumor necrosis factor
TSS	Transcription start site
V	Valine (Val)
W	Tryptophan (Trp)
WNT	Wingless-type
wt	Wild type
X	Stop
Y	Tyrosine (Tyr)

General introduction and outline of this thesis

General introduction and outline of this thesis

Elucidation of genetic disorders is important for clinical practice and basic science. It reveals new insights of key mechanisms i.e. in development, metabolism and function. This thesis describes the molecular and functional genetics of the transcription factor *p63* gene (alias *Tumor protein p63*, *TP63* or *Tumor protein p73-like, TP73L*). Heterozygous mutations in the *p63* gene can cause five different human ectodermal dysplasia syndromes and two non-syndromic conditions: Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC, OMIM 604292), Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), Limb mammary syndrome (LMS, OMIM 603543), Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285), Rapp-Hodgkin syndrome (RHS, OMIM 129400), Split hand/foot malformation (SHFM4, OMIM 605289) and Non-syndromic cleft lip/palate (NSCL). Common characteristics of these p63-associated diseases are abnormalities in ectodermal derived structures, limb malformations and orofacial clefting. I will summarize the present knowledge about the development of these tissues and structures, and discuss disease conditions overlapping the p63-associated disorders. Finally, I will describe the *p63* gene and its function, and provide an outline of the work presented in this thesis.

1.1 Ectodermal development

Gastrulation is the stage in the early embryonal development when cells are organized into three different germ layers: endoderm, mesoderm and ectoderm. These three primary germ layers consist of progenitors of all cellular and tissue lineages of the body, except the germ cells (Fig. 1). Each germ layer will differentiate and give rise to different organs, following series of specific signaling programs. The most inner endoderm forms respiratory and digestive tubes. The middle mesoderm layer forms connective tissue (blood, cartilage and bone cells), skeletal and heart muscle, the uro-genital system and parts of the internal organs. The outer ectoderm layer gives rise to skin, its appendages and nervous system. The ectoderm will differentiate into ventral epidermis and dorsal neural zone following signaling induction of fibroblast growth factor (FGF), Bone morphogenetic protein (BMP) and BMP antagonist (1,2). The neural zone comprises the neural plate and at later stages the neural tube, which will separate from the overlying epidermal ectoderm and give rise to the brain and the spinal cord. The cells connecting the neural tube and ectoderm will become neural crest cells and will generate peripheral neurons, glia-cells, skin pigment cells and several other cell types (3). Finally, the epidermal ectoderm will differentiate into stratified skin and its derivatives, which will be discussed later in this chapter. The main focus of interest of this thesis is the morphogenesis and differentiation of epidermal ectoderm that is altered in human ectodermal dysplasias.

Molecular signaling during human embryonal development and differentiation is complex. Dissection of the individual signaling pathways and the interplay between them is a challenging task. In addition, most of the studies have to be conducted in model organisms that may have a different structure or development than the organ of interest in humans. Nevertheless, animal-based studies, especially transgenic and conditional knock-out mouse models have greatly impacted our knowledge of the basic mechanisms of embryonal development and the genes involved in it. These studies have shown that the signaling between the developing epithelium and underlying mesenchyme is crucial for the correct induction, morphogenesis and differentiation of epidermis and its appendages (4).

Zygote

↓

Cleavage

↓ → Primordial germ cells → Gametes

Gastrulation

ECTODERM

→ Lens of the eye

→ Auditory vesicle* → Inner ear mechanism

→ **Outer epithelium of the body**

→ Mammary glands, Sweat glands, Sebaceous glands, Nails, Hair

→ Nasal and olfactory epithelium and olfactory nerve

→ Stomodeal epithelium → Enamel of teeth, Oral epithelium, Anterior lobe of hypophysis

→ Proctodeal epithelium → Anal canal*

→ **Neural tube**

→ Motor spinal nerve roots, Spinal cord, Pars neuralis of hypophysis, Brain, Cranial motor nerves, Optic vesicles → Retina* and optic nerve

→ **Neural crest**

→ Cranial sensory ganglia and nerves

→ Spinal dorsal root ganglia → Sensory spinal nerve roots, Medulla of adrenal, Sympathetic ganglia

→ Dentine of teeth

→ Skull and branchial cartilages, Cephalic connective tissue (together from head mesenchyme)

MESODERM

→ **Notochord**

→ **Dorsal mesoderm**

→ Sclerotomes → Axial skeleton

→ Myotomes → Appendicular skeleton, Muscles of appendages

→ Skeletal muscles of trunk

→ Dermatomes → Connective tissue layers of skin

→ **Intermediate mesoderm**

→ Mesonephric ducts → Epidymis, vas deferens

→ Metanefric diverticulum, Ureters, Renal pelvis, Collecting tubules

→ Mesonephros, Efferent ducts

→ Metanephros, Kidney tubules*

→ Müllerian ducts → Vagina*, Oviducts*, Uterus*

→ Pronephros

→ **Lateral mesoderm**

→ Somatic mesoderm → Pleura, Pericardium, Peritoneum

→ Splanchnic mesoderm:

→ Mesenchyme → Stroma of gonads

→ Connective tissue and smooth muscle of viscera and blood vessels

→ Hemangioblastic tissue → Blood corpuscles, Endothelium of blood vessels, Endocardium

→ Mesenteries, Visceral pleura and peritoneum, Epimyocardium, Epicardium, Myocardium (Heart)

→ **Head mesenchyme**

→ Outer layers of eye

→ Muscles

ENDODERM

→ Primitive gut → Trachea*, Bronchi, Lungs

→ Digestive tube* → Pancreas*, Liver, Allantois* → Urinary bladder*

→ Pharynx → Thyroid

→ Pharyngeal pouches* → Middle ear*, Tonsillar recesses*, Primitive thymus, Parathyroids*

* Origin of epithelial part of organ only, secondary supporting investments of mesodermal origin.

Figure 1. Gastrulation gives rise to the three primary germ layers: endoderm, mesoderm and ectoderm. These primary germ layers consist of progenitors of all cellular and tissue lineages in the body, except the germ cells. Primordial germ cells separate from the zygote after the cleavage just prior to the gastrulation.

Although the signaling cascades are not fully understood, a number of pathways such as Transforming growth factor-β (TGFβ) including Bone morphogenetic proteins (BMP), Fibroblast growth factor (FGF), Wingless-type (WNT), Sonic hedgehog homolog (SHH), Tumor necrosis factor (TNF) signaling and several transcription factors have crucial roles in epidermal development and differentiation (5-7).

Epidermis

Embryonic epithelium is initially a single cell layer of multipotent epithelial cells that covers the human embryo. Due to an unknown initiation event, these cells start to proliferate and produce a temporary outer single-cell layer of simple squamous cell epithelium, called periderm. Cells in the inner layer become then slow-cycling basal stem cells (SC) with a high proliferative capacity (Fig. 2). Stratification of the epidermis begins by an asymmetric cell division, where one daughter cell retains the stem cell capacity and the other becomes a rapidly proliferating transit amplifying cell. After a few cell divisions transit amplifying cells detach from the basal lamina, migrate upwards and start their differentiation program. The transit amplifying cells that detach the basal lamina become spinous cells and constitute the *stratum spinosum*, a layer of keratin filament network. When spinous cells differentiate the keratin accumulates in granules in granular cells that constitute the cornified envelope, *stratum granulosum*. Finally, these granular cells transit to the *stratum corneum* that is a dead upper layer where all transcriptional and metabolic activities have ceased. The underlying basal lamina and stem cell layer, *stratum basale*, form together with these three differentiation layers the epidermis, which protects the human body from physical and chemical stress and prevents it from dehydration and infections caused by micro-organisms.

The embryonic ectoderm not only produces a stratified epidermis, but also several ectodermal appendages, such as hair, teeth, nails and a number of glands (e.g. sweat, sebaceous and mammary glands). These derivatives originate upon reciprocal signaling between the ectodermal epithelium and underlying mesenchyme (7). The interactive signaling that can originate either from the mesenchyme (hair and mammary gland) or neural crest (teeth and cranial hair) results in proliferation and clustering of epidermal keratinocytes causing ectodermal thickenings.

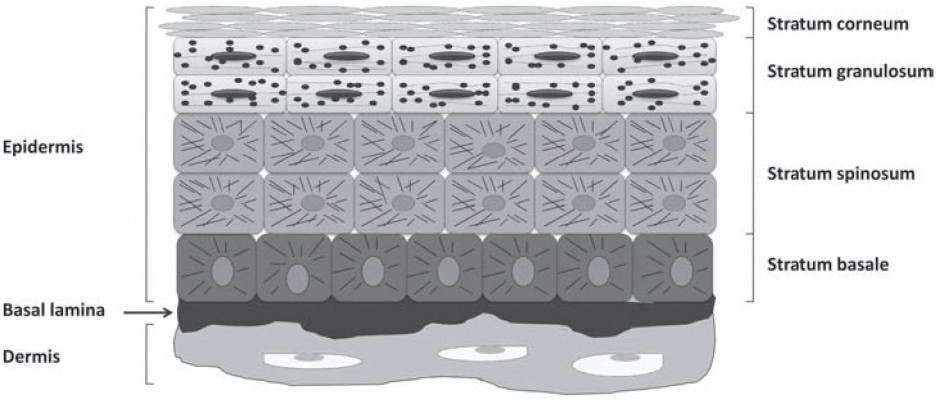

Figure 2. Epidermis. Epidermis is the outer part of human skin and lies on the dermis and basal lamina. Epidermis consists of four layers stratum basale, stratum spinosum, stratum granulosum and stratum corneum.

The resulting epidermal placode then signals back to underlying dermis, which causes an aggregation of mesenchymal cells and give rise to a bud that invaginates into the dermis. The beginning of the dialogue between epidermis and dermis is highly similar in the development of the various ectodermal appendages; however, the subsequent signaling events are more specific for each individual structure.

Hair and sebaceous gland development

Hair is the first specialized structure that differentiates from the embryonic ectoderm. Hair follicle development immediately follows the skin stratification and may play an important role in initiating barrier formation (8). Mesenchymal WNT, FGF and BMP inhibitory factors start the signaling crosstalk and together with epidermal WNT signaling give rise to placode formation and bud invagination (4). The hair bud begins to proliferate and becomes a rod like hair peg, of which dermal tip broadens. Also dermal cells proliferate and, due to SHH signaling, they form a dermal papilla underneath the hair bulb (4). The hair bulb consists of highly proliferative matrix cells. Following a growth stimulus from the dermal papilla the matrix cells exit the cell cycle, move upward and differentiate into the hair shaft or inner root sheath (Fig. 3). Alongside the hair sheath is a bulge, which contains a reservoir of quiescent stem cells. These bulge stem cells become active at the beginning of every new hair cycle and move downward to the hair bulb to produce matrix cells (9). Above the bulge in every hair shaft is a sebaceous gland, which produces and secretes lipid-rich sebum into the hair canal that empties it out to the skin surface. Only little is known about sebaceous gland formation. However, it has been shown that the sebaceous gland can originate both from multipotent bulge stem cells and from its own progenitors, which exist within the sebaceous gland itself (9-12). By today several signaling pathways such as WNT, FGF, Nuclear factor κB (NFκB), Notch and BMP have been linked to hair induction and differentiation (4,5).

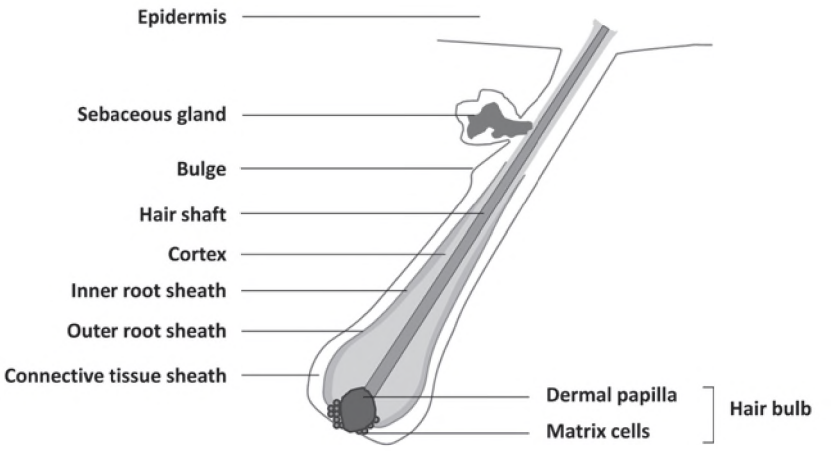

Figure 3. Structure of the hair. Hair shaft is surrounded by cortex, inner and outer root sheath and connective tissue sheath. Underneath the sebaceous gland lies bulge stem cells that give rise to matrix cells, which proliferate and differentiate to a hair shaft and cortex.

Developing teeth

Tooth induction is very similar to hair induction, however the first signals are derived from the oral ectoderm instead of mesoderm (13). The expression of *FGF8, BMP4* and *PITX2* are reported to induce formation of a dental lamina (7,13). Dental lamina is a thickened U-shaped epithelial ridge in the upper and lower jaws where teeth will be formed. This signaling activates mesenchymal transcription factors *(MSX1/2, DLX1/2, GLI2/3)* that initiate bud formation in teeth morphogenesis. Then ectodermal response of *LEF1, EDAR* and four main teeth signaling pathways (BMP, FGF, SHH and WNT) initiate invagination of the bud into the mesenchyme. Mesenchymal factors *MSX1, PAX9* and *RUNX2*, and BMP and FGF signaling cause mesenchymal cell condensation around the tooth bud and extensive growth of the bud, which is followed by enamel knot induction. Enamel knot is a signaling center that is situated at the tip of the epithelial bud. Furthermore, enamel knot is crucial for transition of the bud to a cap-stage, marking the onset of tooth crown development. Several signaling pathways and *EDAR* are involved in induction and formation of enamel knots and a dental papilla. Dental papilla will be formed under the tooth bud, where it migrates upwards into the dental lamina and constitutes the core of the developing tooth. Finally, terminal differentiation takes place. Mesenchymal cells along the dental papilla will differentiate into dentin producing odontoblasts and dental lamina will differentiate into enamel producing amyloblasts that cover and protect the teeth. BMP, FGF, SHH and WNT signaling pathways and several transcription factors are crucial for teeth development, but in total more than 300 genes have been associated with teeth patterning, morphogenesis and differentiation. (7,13,14)

Mammary gland development

Mammary gland is the third structure that is initiated by extensive epithelial-mesenchymal interactions and that follows the tooth and hair follicle development. Response to the first mesenchymal signals cause thickening of the epidermal cells and give rise to two mammary ridges in the ventral epidermis. Placodes develop at the site of developing mammary gland, while the rest of the mammary line disappears in human. Several factors, such as *TBX3* transcription factor, FGF and WNT signaling are reported to be important regulators of placode development (15,16). WNT signaling and *MSX1/2* are needed for placode invagination into the dermis and also for primary bud formation (15). Parathyroid hormone related protein (PTHrP) induces the formation of mammary mesenchyme, and together with its receptor PTH1R and BMP4 it regulates mammary bud branching upon contact with fat pad and formation of secondary bud-structures that lengthen and form lactiferous ducts (17). These ducts open into an epithelial mammary pit, which will later differentiate into a nipple. The main pathways known to be involved in embryonic mammary gland development are WNT and FGF signaling, but recently also Hedgehog homolog signaling (HH) and *GATA3* have been implicated (17).

Development of other ectodermal structures

Besides the described ectodermal appendage development also numerous other embryonal organs and structures arise from the ectoderm. Formation of the placode, the local ectodermal thickening and the reciprocal interactions between mesenchyme and epiderm are common for the initiation and morphogenesis of all of these structures. The head and facial region contain several structures

Table 1. Ectodermal dysplasia syndrome can be divided into four different categories: (1) cell-cell communication and signaling, (2) cell junctions and intermediate filaments, (3) transcription regulation and (4) chromosome stability.

Group 1. Cell-cell communication and signaling.

Disease	OMIM	Inheritance	Gene	Protein	Function	Pathway	Specific symptoms	Reference
EDA (ED1)	305100	XR	*EDA*	Ectodysplasin-A	Ligand	NFκB, TNFα, JNK/c-fos/c-jun	Hypohidrotic ED	(25)
Hypodontia	300606	XR	*EDA*	Ectodysplasin-A	Ligand	NFκB, TNFα, JNK/c-fos/c-jun	Nonsyndromic hypodontia	(29,30)
ED3	129490	AD	*EDAR*	EDAR	EDA receptor	NFκB	Hypohidrotic ED	(26)
HED	224900	AR	*EDAR*	EDAR	EDA receptor	NFκB	Hypohidrotic ED	(26)
HED	224900	AR	*EDARADD*	EDARADD	Adapter	NFκB	Hypohidrotic ED	(27)
ED3	129490	AD	*EDARADD*	EDARADD	Adapter	NFκB	Hypohidrotic ED	(28)
HED-ID	300291	XR	*NEMO*	IKKγ	IκB phosphorylating kinase complex	NFκB	Hypohidrotic ED, ID	(33)
IP	308300	XD	*NEMO*	IKKγ	IκB phosphorylating kinase complex	NFκB	Incontinentia pigmenti (familial male lethal type)	(34)
OL-EDA-ID	300301	XD	*NEMO*	IKKγ	IκB phosphorylating kinase complex	NFκB	Hypohidrotic ED with osteopetrosis, lymphoedema, ID	(35)
EDA-ID	-	AD	*IKBA*	NFKBIA	NFκB inhibitor complex	NFκB	ED, T-cell ID	(36,38)
SMMCI	147250	AD	*SHH*	Shh	Signal molecule	Shh	Fused incisors, midline developmental defects, craniofacial anomalies	(39,40)
LADD	149730	AD	*FGFR2*	Fgfr2	Receptor	Fgf	ED, ear anomaly, hearing loss	(134)
LADD	149730	AD	*FGFR3*	Fgfr3	Receptor	Fgf	ED, ear anomaly, hearing loss	(134)
LADD	149730	AD	*FGF10*	Fgf10	Ligand	Fgf	ED, ear anomaly, hearing loss	(134)
ALSG	180920	AD	*FGF10*	Fgf10	Ligand	Fgf	Lacrimal and salivary gland malformation	(135)
OODD	257980	AR	*WNT10A*	WNT10A	WNT regulator	WNT	ED, smooth tongue, keratoderma, hyperhydrotic palms and soles	(136)
Focal dermal hypoplasia	305600	XD	*PORC*	Porcupine	Membrane bound ER protein	WNT	Dermal hypoplasia, papillomas, limb malformation, eye problems	(137)
Hypotrichosis	604379	AR	*LIPH*	LIPH	Lipase H	LPA pathway	Nonsyndromic hypotrichosis	(138)
Localized hypotrichosis	611452	AR	*P2RY5*	P2Y5	Orphan G-protein-coupled receptor	LPA pathway	Nonsyndromic isolated alopecia	(139)

Group 2. Cell junctions and intermediate filaments.

Disease	OMIM	Inheritance	Gene	Protein	Function	Pathway	Specific symptoms	Reference
CLPD1 (ED4)	225060	AR	*PVRL*	Nectin-1	Cell-cell adhesion molecule	NAP cell adhesion	ED, CL/P, limb defects or non-syndromic orofacial clefting	(140,141)
ED-skin fragility syndrome	604536	AR	*PKP1*	Plakophilin-1	Desmosomal protein	Armadillo protein	ED with severe skin fragility	(44-46)
Naxos disease	601214	AR	*PKGB*	Plakoglobin	Desmosomal protein	Armadillo protein	ARVC, palmoplantar keratoderma, woolly hair	(47)

that are of ectodermal origin. The eye lens is induced by neuro-ectodermal contact with ectoderm, the cornea develops from the ectoderm that overlays the lens and finally the eyelids form as the lens detaches from the ectoderm (18). The cochlea is a sensory organ in the inner ear for balance and hearing, which originates from endo-mesodermal and ectodermal signaling (19). Also part of facial morphogenesis is initiated by local ectodermal thickenings that comprise later the nasal and oral cavities. Furthermore, many eccrine glands such as salivary, lacrimal and sweat glands are all derivatives of the ectoderm. Limb development relies on the apical ectodermal ridge (AER), a signaling center controlling limb development and outgrowth. Finally also nails are of ectodermal origin. (3)

1.2 Ectodermal dysplasia syndromes

Ectodermal dysplasias (ED) are a large and heterogenous group of diseases that are characterized by abnormal development or growth of tissues and structures that originate from the outer embryonal layer, the ectoderm. A condition is called ED, when two or more of the following structures are affected: skin, hair, teeth, nails or sweat glands. Also conditions that combine a defect in one of those structures with an anomaly of another ectodermal structure are referred to as ectodermal dysplasia. ED patients have often dry, fine and hypopigmented epidermis. There can be scaling at birth or patches of hyperkeratosis, eczema or dermatitis resembling atopic skin disease. Abnormalities in hair follicles can give rise to sparse, curly and blond hair. Hypotrichosis or increased hair fragility can lead to alopecia. The amount of body hair follicles can be diminished, and eyelashes and eyebrows may be malformed or even absent. Dental changes can be seen in malformed and absent teeth. In addition, enamel dysplasia and salivary gland malfunction predispose the teeth for caries. Therefore teeth are often replaced by prosthesis already at young age. Nails can be dystrophic, abnormally keratinized, thickened, discolored and fragmented. Several glands can be affected. Impaired sweat gland function causes absent or reduced sweating, which can lead to life-threatening hyperthermia. Mammary glands and nipples can be hypoplastic or absent. Lacrimal and meibomian glands of the eye can be malformed causing dry eyes and recurrent infections. Other eye-related symptoms can be corneal dysplasia and cataracts. The above described symptoms can be seen in various combinations in ectodermal dysplasias, but can also be linked to other congenital abnormalities, such as orofacial clefting, limb malformations, diminished immune defense, hearing impairment, renal abnormalities, or mental retardation, and are subsequently termed ectodermal dysplasia syndromes (20,21).

By now about 170 different ED conditions have been described. However, each individual condition is rare on its own since the estimated cumulative incidence is seven in 10 000 births (21). In the past years several clinicians and researchers have established ectodermal dysplasia classifications. The first classification was purely based on clinical manifestations (22), whereas subsequent classifications took the molecular function of the pathogenic gene into account as well (20,23). Since an increasing number of EDs has been linked to gene mutations, it has become feasible to group these genes into five categories: genes that are involved in (1) cell-cell communication and signaling, (2) adhesion, (3) transcription regulation, (4) development and (5) others (24). This classification by Lamartine allows clustering of similar genes and starts to connect these into several common pathways and functional mechanisms. For example *EDA, EDAR, EDARADD, IKK*γ and *IκBA* are all members of NFκB pathway (see below). However, it should be noted that the classification is ambiguous as several genes may fit into two categories at the same time.

Table 1 *(Group 2 continues)*

Disease	OMIM	Inheritance	Gene	Protein	Function	Pathway	Specific symptoms	Reference
Localized hypotrichosis	607903	AR	*DSG4*	Desmoglein-4	Desmosomal protein	Desmosome molecule	Nonsyndromic hypotrichosis	(48)
Hypotrichosis simplex, scalp	146520	AD	*CDSN*	Corneodesmosin	Glycoprotein		Nonsyndromic isolated alopecia	(49)
EEM	225280	AR	*CDH3*	P-cadherin	Adherin junction		ED, macular dystrophy, ectrodactyly	(43)
HJMD	601553	AR	*CDH3*	P-cadherin	Adherin junction		ED, macular degeneration, early blindness	(81)
Vohwinkel syndrome	604117	AR	*LOR*	Loricrin	Cross-linked cell envelope		Mutilating keratoderma, ichtyosis	(142)
Palmoplantar keratoderma	148350	AD	*GJB2*	Connexin 26	Gap-junction		Palmoplantar keratoderma, deafness	(50,51)
KID syndrome	148210	AD	*GJB2*	Connexin 26	Gap-junction		Keratitis, ichtyosis, deafness	(52,54)
HID syndrome	602540	AD	*GJB2*	Connexin 26	Gap-junction		Ichtyosis, hystrix-like, deafness	(53)
Vohwinkel syndrome	124500	AD	*GJB2*	Connexin 26	Gap-junction		Keratopachydermia, constrictions of fingers and toes, cong. deafness	(55)
ED2	129500	AD	*GJB6*	Connexin 30	Gap-junction		Hidrotic ED (normal teeth)	(56)
ODDD	164200	AD	*GJA1*	Connexin 43	Gap-junction		Oculo-dento-digital (isolated syndactyly type III?)	(57-59)
ED hair-nail type	602032	AD	*KRT85*	Keratin 85	Type II keratin	Hair keratin	Complete absence of all hair, nail dystrophy all finger/toe nails	(143)
NFJS	161000	AD	*KRT14*	Keratin 14	Type I keratin	Pair of KRT5	ED, hyperpigmentation, absence of dermatoglyphics	(144)
DPR	125595	AD	*KRT14*	Keratin 14	Type I keratin	Pair of KRT5	ED, lifelong hyperpigmentation, absence of dermatoglyphics	(144)
Pachyonychia congenita-1	167200	AD	*KRT6A*	Keratin 6A	Type II keratin	Pair of KRT16	Focal palmoplantar keratoderma, oral leukokeratosis	(145)
Pachyonychia congenita-1	167200	AD	*KRT16*	Keratin 16	Type I keratin	Pair of KRT6A	Focal palmoplantar keratoderma, oral leukokeratosis	(146)
Pachyonychia congenita-2	167210	AD	*KRT6B*	Keratin 6B	Type II keratin	Pair of KRT17	Multiple sebaceous cysts	(147)
Pachyonychia congenita-2	167210	AD	*KRT17*	Keratin 17	Type I keratin	Pair of KRT6B	Multiple sebaceous cysts	(148)
Steatocystoma multiplex	184500	AD	*KRT17*	Keratin 17	Type I keratin	Pair of KRT6B	Multiple sebaceous cysts, no nail changes	(149)

Especially genes that were known to be involved in development (e.g. *MSX1 and SHH*) were categorized into the development group, although the molecular function was either in transcriptional activity (MSX1) or cell signaling *(SHH)*, which both have their own groups. For these reasons, I present a modified and up-dated classification of the ED genes and syndromes. It is based only on the molecular function of the ED genes and it clusters the disease causing genes into four categories: (Group 1) cell-cell communication and signaling, (Group 2) cell junctions and intermediate filaments, (Group 3) transcription regulation and (Group 4) chromosome stability (Table 1).

EDs are caused mainly by mutations in four types of genes that encode proteins that are involved in cell-cell communication and signaling, cell junctions and intermediate filaments, transcription regulation and chromosome instability (Table 1). Proteins that belong to the cell-cell communication group are involved in several signaling pathways, such as TNF, NFκB, FGF, SHH, WNT and Lysophosphatidic acid (LPA) signaling. Three proteins, Ectodysplasin-A (EDA), its receptor EDAR and an adapter signaling molecule EDARADD, are members of TNFα signaling family and are known to cause identical hypohydrotic ED phenotypes (25-28). Furthermore mutations in *EDA* can cause non-syndromic hypodontia (29,30). Activation of EDA signaling often leads to NFκB activation. However activation of NFκB is preceded by phosphorylation of the NFκB inhibitor IκB by the IKK kinase complex (27,31,32). Interestingly also mutations in the *IKK* kinase and NFκB inhibitor complexes cause ED. Mutations in *IKK*γ give rise to two different types of ED accompanied either with immunodeficiency or a pigmentation disorder (IP) (33-35). Another type of ED with T-cell immunodeficiency is caused by mutations in *IκBA*, a member of NFκB inhibitor complex (36-38). Furthermore, mutations in *Sonic hedgehog homolog (SHH)* gene, which is a downstream target of NFκB, are reported to cause "Solitary median maxillary central incisors syndrome" (SMMCI) characterized by fused incisors and craniofacial anomalies (32,39-41). The defects in various players in one single pathway and its downstream target strengthen the importance of NFκB signaling in the ectodermal appendage development, and suggest that defects in factors in other known ED-associated pathways may also cause ED.

The second group of the EDs consists of genes that encode cell-cell junction and intermediate filament proteins. These types of proteins are important for tissue structure and cell-cell connection and are often seen in mature epithelial cells (Fig. 4). Adherens and desmosomes are the two types of cell-cell anchoring junctions that can be found in epithelial cells. The first one anchors actin filaments and the latter one intermediate filaments in order to connect them with neighboring cells. Mutations in both types of anchoring junction coding genes *(P-cadherin, Plakophilin-1, Plakoglobin, Desmoglein-4, Corneodesmosin)* can cause ED or non-syndromic hair disease (42-49). Gap-junctions are channel-forming junctions in epithelium, which allow small water-soluble molecules to move from cell to cell. Connexins are the major gap-junction proteins, and dominant mutations in three different connexin genes *(CX26, CX30, CX43)* have been reported to lead to different types of ED (50-59). Intermediate filaments, such as keratins create an important group of structural filament system of the cytoskeleton protecting the tissue against physical stress. Keratins are expressed mainly in epithelial cells, however, some keratins can be hair specific. Mutations in six different keratin genes *(KRT6A, KRT6B, KRT14, KRT16, KRT17, KRT85)* are causative for different types of EDs. Interestingly, some of these keratins form pairs together creating a functional keratin filament, thus suggesting that both members of the pair are important, but also that these certain keratin filaments are crucial for the ectodermal development.

Table 1 *(continues)*

Group 3. Transcription regulation.

Disease	OMIM	Inheritance	Gene	Protein	Function	Pathway	Specific symptoms	Reference
EEC	604292	AD	*p63*	TP63	Transcriptionfactor		ED, limb anomalies, CL/P	(74)
AEC	106260	AD	*p63*	TP63	Transcription factor		ED, ankyloblepharon, CL/P	(75)
RHS	129400	AD	*p63*	TP63	Transcription factor		ED, CL/P	(76)
LMS	603543	AD	*p63*	TP63	Transcription factor		ED , limb anomalies	(77)
ADULT	103285	AD	*p63*	TP63	Transcription factor		ED, limb anomalies	(78,79)
Ellis-v-Creveld syndrome	225500	AR	*EVC*	EVC	Nuclear receptor?		Chondro-ED, polydactyly, heart defects	(84)
Ellis-v-Creveld syndrome	225500	AR	*EVC2*	EVC2	Nuclear receptor?		Chondro-ED, polydactyly, heart defects	(85)
Weyers acrofacial dysostosis	193530	AD	*EVC*	EVC	Nuclear receptor?		Chondro-ED, hypotelorism, polydactyly	(84)
Weyers acrofacial dysostosis	193530	AD	*EVC2*	EVC2	Nuclear receptor?		Chondro-ED, hypotelorism, polydactyly	(86)
T-Cell ID, alopecia and nail dystrophy	601705	AD	*FOXN1*	FOXN1	Transcription factor		ED, alopecia, T-cell ID	(69)
Nail dystrophy	-	AR	*FOXN1*	FOXN1	Transcription factor		Nonsyndromic nail dystrophy	(70)
Bamforth-Lazarus syndrome	241850	AR	*FOXE1*	FOXE1	Transcription factor	Forkhead/winged helix family	Hypothyroidism, thyroidal, choanal atresia, spiky hair, CL/P	(71)
Atrichia with popular lesions	209500	AR	*HR*	Hairless	Nuclear receptor repressor	WNT	Hair loss after birth, follicular keratinous cysts	(81)
Alopecia universalis congenita	203655	AR	*HR*	Hairless	Nuclear receptor repressor	WNT	Nonsyndromic alopecia	(82,83)
Tricho-dento-osseous (TDO)	190320	AD	*DLX3*	Distal-less-3	Transcription factor	Homeobox gene	ED and bone anomalies	(66,68)
AIHHT	104510	AD	*DLX3*	Distal-less-3	Transcription factor	Homeobox gene	Amelogenesis imperfecta, taurodontism	(67)
Selective tooth agenesis	604625	AD	*PAX9*	Pax9	Transcription factor	Paired box family	Nonsyndromic tooth agenesis	(60-62)
Tooth agenesis	106600	AD	*MSX1*	Msx1	Transcriptional repressor	Homeobox gene	Nonsyndromic tooth agenesis, sometimes with orofacial clefting	(63,64)
Witkop syndrome	189500	AD	*MSX1*	Msx1	Transcriptional repressor	Homeobox gene	Tooth and nail syndrome	(65)

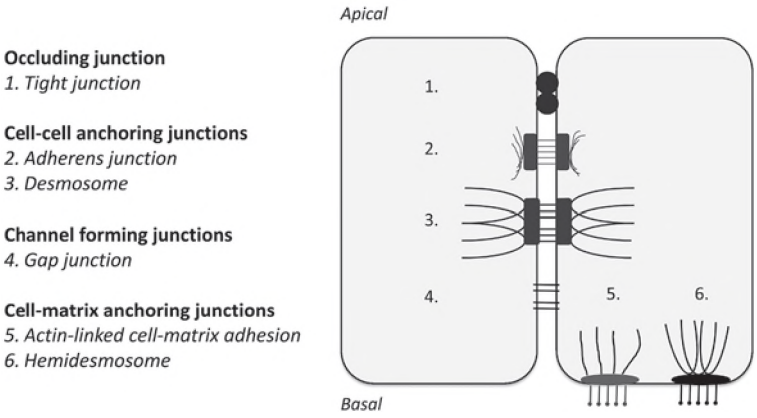

Figure 4. Cell-cell junction and intermediate filament proteins in epidermis.

The third ED group consists of nine genes that regulate transcription. Generally, mutations in ED causing genes can often cause several allelic conditions, because the type of mutation dictates the inheritance pattern and phenotype, but also probably due to different genetic background. In some conditions also the way of inheritance modifies the phenotype, e.g. homozygous mutations in EVC gene gives rise to Ellis van Creveld syndrome (OMIM 225500), whereas heterozygous mutations lead to Weyers acrofacial dysostosis (OMIM 193530). ED causing defects have been reported in genes from paired box *(PAX9)*, homeobox *(MSX1, DLX3)* and forkhead/winged helix domain gene families *(FOXE1, FOXN1)* (60-71). Interestingly, certain mouse models of other members of these gene families also show aberrations in ectodermal development suggesting their importance in development, but also as ED candidate genes (72,73). Dominant mutations in p63 transcription factor gene also lead to several ED syndromes (74-79). Not only defects in transcription factors, but also in nuclear receptor co-repressor gene *Hairless* and possible nuclear receptors *EVC1/2* cause ED (80-86). The large amount of mutated transcription factors causing ED indicates the important role of transcription regulation in ectodermal development.

The fourth group of genes regulates the chromosome stability. Five different genes are known to cause Dyskeratosis congenita, which is characterized by nail dystrophy, abnormal skin pigmentation, leucoplakia, bone marrow failure and predisposition to cancer (87). Four mutated genes *DKC1, TERC, TERT* and *NOLA1* are components of telomerase complex, and it is believed that telomerase deficiency leads to chromosome instability (87-92). The fifth Dyskeratosis congenita causing gene *TINF2* is a member of telomere protecting complex, shelterin (93). This "chromosome stability" group differs clearly from the rest three ED classification clusters that are linked together in a way that junctions allow cells to create pathways for communication and signaling, which then regulate the gene expression.

1.3 Transcription factor *p63*

Yang et al discovered a new member for the *p53* and *p73* gene family in 1998 (94). The new member *p63* (also known as *TP63, KET, Tp73L, p40*) turned out to be the ancestral member of this transcription factor gene family (94,95). It is located on the long arm of the chromosome 3 (3q27-28) and consists in total of 16 exons. At least six different p63 isoforms can be produced, due to

Table 1 *(continues)*

Group 4. Chromosome stability.

Disease	OMIM	Inheritance	Gene	Protein	Function	Pathway	Specific symptoms	Reference
Dyskeratosis congenital	305000	XR	*DKC1*	Dyskerin-1	Small nucleolar ribonucleoprotein	Component of telomerase complex	Bone marrow failure, leucoplakia, abnormal skin pigmentation, nail dystrophy, predisposition to cancer	(88,89)
Dyskeratosis congenital	224230	AR	*NOLA3*	NOLA3	Small nucleolar ribonucleoprotein	Component of telomerase complex	Bone marrow failure, leucoplakia, abnormal skin pigmentation, nail dystrophy, predisposition to cancer	(90)
Dyskeratosis congenital	127550	AD	*TERT*	TERT	Ribonucleoprotein polymerase	Component of telomerase complex	Bone marrow failure, leucoplakia, abnormal skin pigmentation, nail dystrophy, predisposition to cancer	(91)
Dyskeratosis congenital	127550	AD	*TERC*		snoRNA (Telomerase RNA)	Component of telomerase complex	Bone marrow failure, leucoplakia, abnormal skin pigmentation, nail dystrophy, predisposition to cancer	(92)
Dyskeratosis congenital	127550	AD	*TINF2*	TRF1	DNA binding protein	Component of shelterin complex	Bone marrow failure, leucoplakia, abnormal skin pigmentation, nail dystrophy, predisposition to cancer	(93)

two different promoter sites and three different splicing routes. The amino-terminal ends are encoded either by exons 1-3 or exon 3' only, and are called TA and ΔN, respectively (Figure 5A). The carboxy-terminal ends denoted α, β and γ are result of alternative splicing routes for exons 10-15. Several functional domains have been identified in p63 protein. The canonical transactivation domain (TA) bears homology to the TA domain of p53 and is present only in the TA-isoforms. However, ΔNp63 isoforms which lack this TA domain exhibit nonetheless transactivation activity and it has been demonstrated that the amino terminus of ΔN contains a second TA domain (TA2) (Fig. 5B) (96,97). The DNA binding domain (DBD) is present in all isoforms and is crucial for the binding on to its target promoter. The isomerization domain is needed for dimerization of p63 molecules and is present in all isoforms. The carboxy-terminal end has two additional domains: sterile alpha motif domain (SAM) and transactivation inhibitory domain (TI). The SAM domain is involved in protein-protein interactions and is present only in the longest α-isoform. The TI domain that is found in α- and β-isoforms, is able to bind to the amino-terminal TA domain intramolecularly and inhibit its function (98).

Role of p63 in development

At the time *p63* was found, it was anticipated that it has tumor-suppressor properties, just as its famous family member *p53*. However, several studies provided unequivocal evidence that p63 has a major role in embryonal development: *p63* knock-out (KO) mouse models showed an extreme form of developmental abnormalities and dominant *p63* mutations in human led to congenital ectodermal dysplasia syndrome (74,99,100).

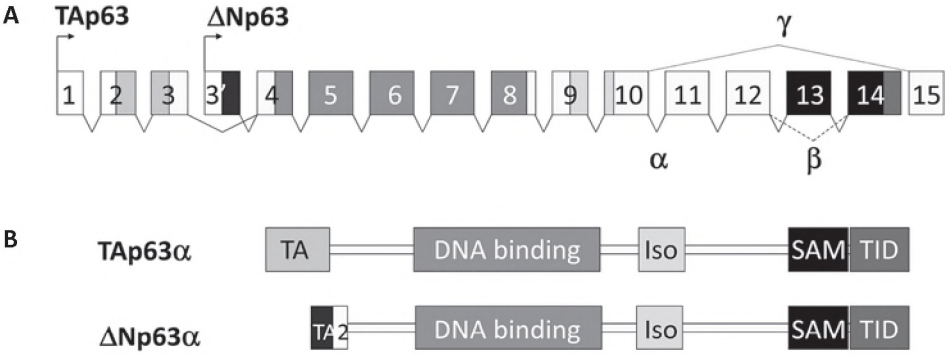

Figure 5. Schematic overview of the *p63* gene and two of its protein products. A. *p63* has two different promoter sites resulting in two different N-terminal ends called TA and ΔN. ΔNp63 lacks the exons 1-3, which are present in TAp63, however it contains an alternative exon 3'. *P63* has three C-terminal splicing routes: α, β and γ, thus at least six different gene products can be transcribed. **B.** The domain structure of TA and ΔNp63 is similar, only N-terminal transactivation domain is different, called TA and TA2, respectively. Domain abbreviations: ISO (Isomerization domain), SAM (Sterile alpha motif domain) and TID (Transactivation inhibitory domain). Figure adapted from Chapter 6.

By now, mutations in *p63* gene in human can cause seven different diseases: Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome (EEC, OMIM 604292), Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), Limb mammary syndrome (LMS, OMIM 603543), Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285), Rapp-Hodgkin syndrome (RHS, OMIM 129400), Split hand/foot malformation (SHFM4, OMIM 605289) and Non-syndromic cleft lip/palate (NSCL) (74-79,101). There are three main hallmarks that characterize these disease phenotypes: ectodermal dysplasia, orofacial clefting and limb malformations (ectrodactyly and syndactyly). The *p63*-deficient mice phenotype is reminiscent of the human p63-mutant syndrome, though it is more severe. *P63*-KO mice were born with truncated or absent limbs, severe craniofacial hypoplasia, absent hair follicles, teeth inducements and several eccrine glands. These mice basically lacked all stratified epithelia and the epidermis, which caused dehydration and early dead. The mutant mouse studies together with the human disease phenotypes proved that *p63* is a crucial developmental gene especially in the ectodermal development including the limbs and craniofacial parts.

p63: Master molecule of stem cell identity, differentiation or both?

The *p63*-deficient mice were created and studied by two separate research groups, whose interpretations of the defects in the mouse mutants were not consistent (99,100). One of the groups claimed that p63 is required for the commitment of the simple ectoderm to the epidermal lineage, because the group was unable to detect any sign of differentiation (100). In contrast, the other group observed some patchy epidermal differentiation and concluded from their findings that instead of block the induction of stratified epithelia, p63 has main role in stem cell proliferation and maintenance (99). A detailed characterization of the stratification defects in the *p63*-deficient mice revealed that dental lamina, an initiation step in teeth development was normal, although it did not proceed into the teeth placode, whereas the hair follicle development was not even initiated (99,100,102). It is unclear whether strain-specific factors may have affected the phenotype, or whether only different interpretation of the very same phenotype created the discrepancy in the final conclusions. The crucial but controversial role of p63 in ectodermal stem cells inspired several research groups around the world to study the role of p63 and started an interesting and extremely active research period on p63.

Several further studies have been performed in order to resolve the primary role of *p63*. P63 has been shown to be highly expressed in epithelial cells that are highly clonogenic and have high proliferative capacity, whereas cells lacking the *p63* start losing their proliferative capacity (103). Furthermore, adding p63 to primary keratinocytes when inducing the Ca^{2+}-dependent differentiation prevents the keratinocyte differentiation (104,105). Ample evidence exists that p63 is the master molecule that determines the proliferative potential of stem cells in stratified epithelia. However, several groups have also shown that p63 is necessary for epidermal cell lineage commitment. Adding p63 into embryonal stem cell (ES) derived ectodermal cells (potential precursors of the epidermal cells) increased the cell proliferation and survival as well as differentiated these cells into keratinocytes (106). Another study showed that excess p63 was able to induce epidermal stratification markers in single layered lung epithelia, suggesting the role of p63 also in epidermal cell lineage commitment (105). Thus, evidence for both roles of p63 is available, which strongly indicates that p63 is not only important for the stem cell maintenance and proliferation but also for the epidermal stem cell commitment.

p63 isoforms in developing epidermis

Although, independent results support the role of p63 in epidermal cell lineage commitment, the results are not consistent. There is a discrepancy between the expression of different p63 isoforms. It has been claimed that TAp63 isoforms can be detected before the commitment to epidermal stratification at embryonal day 7.5 (E7.5) (105). In the same study it was suggested that TAp63 starts the stratification and inhibits the terminal differentiation and expression of ΔNp63 isoforms (expression at E9.5). The counterbalanced expression by ΔNp63 isoforms would thereby allow the epidermis to differentiate (105). In all other epithelial cell studies the ΔNp63 isoforms appear to be the isoform with highest expression (102,106-108). Its expression has been detected first at around E8, whereas in only one study very small amounts (0.8 percent) of TAp63 isoforms have been detected (E13) (102). In contrast to Koster's study (105), the TAp63 expression has been detected to increase and ΔNp63 decrease during the keratinocyte differentiation (109). Results of isoform specific complementation in *p63*-deficient mice revealed that mice complemented by ΔNp63α (lacking the TAp63α) showed some differentiated epidermal structure, whereas mice complemented by TAp63α (lacking the ΔNp63α) had only poorly differentiated keratinocyte patches (107). Yet, the epidermis of the mice lacking ΔNp63α is very similar to the complete *p63* KO-mice. Interestingly, TA-specific KO-mice had normal epithelial morphogenesis, but showed defects in genomic surveillance in oogenesis (110). Altogether this data strongly demonstrates that ΔNp63α is the crucial p63-isoform that is first expressed in the epidermis.

p63 regulates several developmental genes

By now several interesting developmental p63 target genes have been discovered. ΔNp63α has been shown to bind to the *GATA-3* promoter and activate the gene transcription (111). GATA3 is a transcription factor that is involved in skin and hair follicle cell fate decision (109,111,112). Interestingly, GATA3 can then regulate IκB kinase-α *(IKKα)* that has been linked to epidermal differentiation, proliferation and limb development (111,113,114). However, also direct regulation of *IKKα* has been reported by TA/ΔNp63 (111,115). Recently, TA/ΔNp63 has been demonstrated to directly regulate three members of *Distal-less homeobox* gene family *(DLX3, DLX5 and DLX6)* that are linked into the development of head and limb skeleton (116,117). P63 does not only regulate genes by activating or repressing their transactivation but also through protein-protein interactions. The SAM domain of p63 can bind to Apobec1-binding protein 1 (ABBP1) that is a member of RNA processing machinery and known to regulate differential splicing of the Fibroblast growth factor receptor 2 (FGFR2) towards the epithelial specific form (118). Very likely p63 and SAM domain have other interacting proteins that have not been discovered yet.

The amount of p63 direct and indirect target genes increases rapidly. The difficulty is to discover their contribution to the development when some of these targets are not expressed in epithelial or ectoderm-derived tissues. Furthermore, several novel targets have been shown to be activated only by TAp63α isoform that is not expressed in epithelial or ectodermal derived tissues or structures. Recently, several research groups have performed genome-wide p63 target gene studies (119-121). Their studies resulted in detection of certain enriched cellular processes that are likely to be regulated by ΔNp63α: development, morphogenesis, proliferation, tissue regeneration, cell adhesion, death and signaling pathways (119,120). Some validated developmental and morphogenic target genes have already been described above, and next to that I will introduce the known adhesion-linked genes that are regulated by p63.

p63 regulates epithelial adhesion

Recently, p63 has been linked to the regulation of epithelial adhesion and survival (108). Since the epithelium is confronted with a lot of mechanical stress, its structure has to be very compact and solid. Cells are bound together by direct cell-cell anchoring junctions (adherens and desmosomes) and cells in the basal layer are anchored to the underlying basal lamina by cell-matrix junctions (actin-linked adhesions or intermediate filament-linked hemidesmosomes) (122) (Fig. 4). The signal transmission is allowed through the actin or intermediate filaments that are anchored to these junctions. P-cadherin, an adherens junction protein can be transcribed and activated by various p63 isoforms (123). Also PERP protein, a stabilizer of desmosomal complexes within cell-cell junctions, can be regulated and activated by various p63 isoforms in stratified epithelia (124). Furthermore, several cell-matrix adhesion proteins have been shown to be regulated by p63 (108,125). Epithelial matrix, the basal lamina consists mainly from laminin molecules, and interestingly a component of a laminin receptor *Integrin-α3* has been shown to be a TAp63γ target (125). Knockdown of endogenous *p63* led to cell detachment from the plate, down-regulation of cell adhesion-associated genes and cell death in human epithelial breast cell line and keratinocytes (108). ΔNp63α was shown to be able to occupy several *Integrin* (α3, β4, α5 and α6) and *Laminin-γ2* promoters and activate their transcription. Thus the detachment of the plate was probably caused by the down-regulation of these adhesion-related genes. It was also shown that the detachment was independent of apoptosis, and that ΔNp63α was important for cell survival. Finally, Dystonin (BPAG1) that anchors keratin containing filaments to hemidesmosomes and basal layer intermediate protein Keratin-14 can be regulated by TAp63γ and ΔNp63α, respectively (107,126-128).

Epithelium also has a selective permeability barrier function, which is formed by tight junctions. Tight junctions seal gaps between epithelial cells to prevent exchange of water, ions and macromolecules across epithelial sheets. Claudins are main transmembrane proteins for the tight junction formation and function. (122,129) The importance of *Claudin-1 (CLDN1)* has been shown by *Cldn1*-deficient mice that lack the tight junctions in the skin epithelia and consequently dye on dehydration (130). Recently, ΔNp63 has been reported to bind *CLDN1* promoter and activate its transcription, indicating that *CLDN1* is a direct target gene of the ΔNp63α (131). The regulation of cell junction and adhesion genes strongly indicate that p63 has a role in cellular adhesion, which can have effects for cell metabolism, proliferation, migration, differentiation, structural organization and cell survival (122,132).

1.4 Aim and outline of this thesis

The work in this thesis was initiated in 2004, five years after the *p63* mutant mice studies and the first human *p63* mutations were published (74,99,100). In these five years, it was discovered that mutations in *p63* gene can lead to six different diseases: EEC, AEC, LMS, ADULT, RHS and SHFM4 (74-79,101). In addition, Non-syndromic cleft lip/palate (NSCL) has been associated to *p63* mutations (101). Although the features in these phenotypes are overlapping they are sufficiently different to be considered as unique disorders. Moreover a clear genotype-phenotype correlation has tentatively been established (133). Nonetheless, the exact molecular disease mechanism of these diseases was still poorly explored. My research focused in delineation of *p63* genotype-phenotype association and exploration of the role of p63 in ectodermal dysplasia syndromes. In this thesis I have tried to provide more insight into the p63-associated diseases, their molecular disease mechanism and the role of *p63* and its downstream signaling in these diseases.

Chapter 2. Spotlight on p63: p63-associated disorders.

This chapter gives an overview of the known five ectodermal dysplasia syndromes and the non-syndromic SHFM4. Also a novel non-syndromic orofacial clefting (NSCL) caused by *p63* mutation will be presented. This study gives an update of known *p63* pathogenic mutations. This chapter has been published as a part of p63 special issue in *Cell Cycle* in 2007 (Spotlight on p63: The emerging p53 family) in order to introduce the p63- associated disorders.

Chapter 3. Pattern of p63 mutations and their phenotypes – update.

This chapter describes the clinical spectrum of 227 patients with *p63* mutations, which confirms the previously presented genotype-phenotype association. However, this phenotype delineation also identifies a large degree of clinical variability in each of the p63-associated disorders. This chapter has been published in *American Journal of Medical Genetics* in 2006.

Chapter 4. Delineation of the ADULT syndrome phenotype due to arginine 298 mutations of the p63 gene.

This chapter delineates the ADULT syndrome genotype-phenotype association. On basis of 16 patients it describes the general phenotype caused by the ADULT syndrome hot spot mutation at arginine (p.R298). Moreover it discusses the relevance of oral squamous cell carcinoma in one patient carrying the heterozygous p.R298 mutation. This chapter has been published in *European Journal of Human Genetics* in 2006.

Chapter 5. Spectrum of p63 mutations in a selected patient cohort affected with Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC).

This chapter presents and confirms the AEC/RHS syndrome genotype-phenotype association. This study introduces one known and ten novel pathogenic *p63* mutations in an American patient cohort. Here we present for the first time that missense mutations occur also in the TI domain. This chapter has been published in *American Journal of Medical Genetics* in 2009 as part of the results of the National Foundation of Ectodermal Dysplasia meeting in Houston, in 2007.

Chapter 6. A novel translation re-initiation mechanism for the p63 gene revealed by amino-terminal truncating mutations in Rapp-Hodgkin/Hay-Wells like syndromes.

This chapter presents completely new insight of AEC/RHS disease model. Here we describe three mutations in the 5' end of the *p63* gene, which lead to premature termination codon. Furthermore, we show that these mutations do not lead to nonsense mediated decay, but to translation re-initiation producing a novel p63 protein variant. This chapter has been published in *Human Molecular Genetics* in 2008.

Chapter 7. Genome-wide profiling of p63 binding sites identifies genes and regulatory elements for p63-related disorders: Elucidation of the genetic basis of Split hand/foot malformation type 1.

This chapter combines a genome-wide p63 binding data with functional gene expression data obtained from keratinocytes in order to discover direct p63 target genes affected in p63-associated patients. Here we discuss p63 binding to regulatory elements, direct target genes that are affected in patients and are contributed to p63-associated phenotypic features. We also report an example of a p63 regulatory element that is found in the SHFM1 locus and controls limb development. *This chapter is under preparation to be submitted.*

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p63-associated disorders

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Abstract

Heterozygous mutations in the transcription factor gene *p63* are causative for several syndromes with ectodermal dysplasia, orofacial clefting and limb malformations as the key characteristics. Different combinations of these features are seen in five different syndromes, of which Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC) is the most common one. Mutations in *p63* can also cause non-syndromic single malformations, such as Split hand/foot malformation (SHFM4) and isolated cleft lip/palate (NSCL). In this article we will present an overview of diseases caused by mutations in the *p63* gene and review the known pathogenic *p63* gene mutations.

Introduction

The transcription factor *p63* is a key regulator of ectodermal, orofacial and limb development. This became apparent in 1999, by the generation of *p63* knockout mice (1,2) and by the finding of dominant mutations in human disorders with ectodermal dysplasia, split hand/foot malformation and orofacial clefting (3-7). Mutations in the *p63* gene can cause at least five different syndromes: Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC, OMIM 604292), Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), Limb mammary syndrome (LMS, OMIM 603543), Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285) and Rapp-Hodgkin syndrome (RHS, OMIM 129400). Furthermore, two non-syndromic human disorders are caused by *p63* mutations: isolated Split hand/foot malformation (SHFM4, OMIM 605289) and recently Non-syndromic cleft lip/palate (8). Here we present an overview of these seven p63-linked conditions, the genotype-phenotype associations and give an update of all known pathogenic *p63* mutations.

p63 phenotype

Ectodermal dysplasia (ED) is one of the three main characteristics of the p63-associated syndromes (Fig. 1). Ectodermal dysplasia manifests as the abnormal development or growth of tissues and structures that are developed from the outer embryonal layer, ectoderm. In this condition skin, hair, teeth, nails and several exocrine glands, such as sweat and sebaceous glands are usually abnormally developed. The epidermis of p63 patients can be very dry, itchy and hypopigmented. In extreme cases widespread areas of the skin can be erosed often including the scalp. This is most common in the AEC syndrome, where patients present patches of life-threatening congenital skin erosion. However, the skin usually recovers after the first year. The amount of scalp and body hair is often diminished and hair can be wiry or curly. Alopecia is sometimes reported. Occasionally the eyelashes and eyebrows are also absent. The number of teeth is often less than in healthy individuals, indicating that there is a reduced number of teeth placodes. Teeth can also be malformed by a conical shape and poor enamel formation, causing subsequent tooth decay. Nails can be dystrophic, thickened and discoloured. The absence or reduced amount of sweat glands is also reported among p63 patients and leads to diminished perspiration, which can be life-threatening. Mammary gland and nipple hypoplasia are other manifestations of the ectodermal dysplasia spectrum observed in p63 patients. Also the development and function of sebaceous and salivary glands are frequently abnormal. Lacrimal duct defects and obstruction of the lacrimal ducts impose a risk for conjunctivitis and corneal damage.

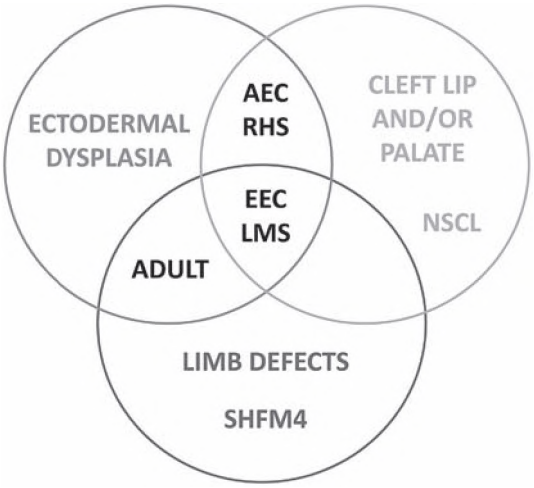

Figure 1. Various combinations of ectodermal dysplasia, orofacial clefting and limb malformations are the hallmark of p63-associated syndromes. EEC syndrome is the prototype of these syndromes and together with LMS shows all three hallmarks. ADULT syndrome patients never show orofacial clefting, whereas AEC and RHS never show limb defects. Non-syndromic limb defect condition (SHFM4) and Non-syndromic cleft lip/palate (NSCL) are also caused by mutations in the *p63* gene.

Characteristics of ED described here varies between the p63 syndromes and even within a one single syndrome, as will be discussed below in this article.

Split hand/foot malformation (SHFM) constitutes the second part of the *p63* syndrome phenotype (Fig. 1). Hands and feet are often malformed and have a severe median cleft in the palm and/or in the sole. These clefts usually occur in conjunction with a lack of one or more central (2-3-4) digits, which is called ectrodactyly. Fusion of fingers or toes, which is called syndactyly, can be seen in conjunction with ectrodactyly. In most cases SHFM is part of a syndrome, but it can also be present in isolation, without other symptoms.

The third hallmark of p63 syndrome phenotype is orofacial clefting (Fig. 1). It is mainly seen in the form of cleft lip (CL) and/or cleft palate (CP). This symptom is usually observed as part of a complex syndrome, where other organs are also affected, however, recently Leoyklang et al (8) have described mutations in *p63* gene causing orofacial clefting as the only abnormality.

Most of the above developmental defects are also observed in *p63* knockout mice, which have particular severe defects of ectodermal structures and the limbs (1,2). Heterozygous $p63^{+/-}$ mouse have no significant developmental abnormalities, suggesting that loss-of-function of one allele is not disease-causing. Recently, it has been reported that a high proportion of $p63^{+/-}$ aging mice develop tumours indicating a role of p63 in tumour suppression (9). However, this observation could not be confirmed in an independent study (10). There is no indication that individuals with heterozygous *p63* mutation are prone to develop tumours.

Allelic p63 conditions

EEC syndrome

The prototype of the p63 syndrome family is the EEC syndrome (Fig. 1). EEC syndrome patients are invariably characterized by one or more features of ectodermal dysplasia, which can present as defects of hair, skin, nails, teeth and glands. The severity and type of the ectodermal features is highly variable, and to some extent dependent on the exact nature of the mutation (see below). Only few patients show defects in all of the described ectodermal structures above. EEC patients occasionally also have mammary gland/nipple hypoplasia (14%) and hypohidrosis (11%). About two-thirds of these patients have ectrodactyly, and syndactyly is also frequent (43%). Cleft lip/palate is present in about 40% of the EEC patients, mostly as CL with or without CP (11).

EEC syndrome is mainly caused by point mutations in the DNA binding domain (DBD) of the *p63* gene (Table 1). Altogether 34 different mutations have been reported, and 20 different amino acids are involved. Only two mutations are outside the DNA binding domain: one insertion (c.1572insA) and one point mutation (p.L563P) in the Sterile alpha motif domain (SAM) (4,11). In earlier studies we found five frequently mutated amino acids: p.R204, p.R227, p.R279, p.R280 and p.R304 in the EEC population, all located in CpG islands (Fig. 2). These five mutations explain almost 90% of the EEC syndrome patients (11,12). The five p63 arginine hotspot mutations and probably also other DNA binding domain mutations that are found in EEC syndrome appear to impair the p63 protein binding to DNA (4). The autosomal dominant inheritance of EEC syndrome suggests that the EEC mutations have a dominant negative effect. However, recent genotype-phenotype analyses for the five hotspot mutations revealed significant differences between the corresponding phenotypes. For instance cleft lip/palate is present in the p.R304 mutation population (80%), whereas p.R227 patients seldom have cleft lip/palate. Syndactyly is completely absent in p.R227 population, while 30-60% of the other hotspot mutation population have syndactyly. Genito-urinary defects are frequently observed in p.R227 mutation population (40%), while significantly less in other populations (11). It thus seems that these hotspot mutations exert specific effects. Such specificity might be brought about by different effects of these mutations on promoters for p63 transcriptional target genes. Alternatively, these hotspot mutations may exhibit gain-of-function effects, similar as for the p53 hotspot mutations (13).

Limb mammary syndrome (LMS)

The LMS phenotype resembles the EEC syndrome phenotype, but the ectodermal manifestations are milder (Fig. 1) (3). A consistent feature of LMS is the mammary gland and/or nipple hypoplasia (100%). Lacrimal duct obstruction and dystrophic nails are frequently observed (59 and 46% respectively), hypohydrosis and teeth defects are detected in about 30%, but other ectodermal defects such as hair and skin defects are rarely detected if at all. About 70% of LMS patients have similar limb malformations as in EEC syndrome, and about 30% orofacial clefting, notably always in form of cleft palate (11).

Mutations in LMS are located in the N- and C-terminus of the *p63* gene (Table 1). A large LMS family (29 affected members) has a point mutation in exon 4, causing a p.G76W substitution in the ΔN-specific putative second transactivation domain (TA2) (3,12,14,15).

Table 1. Pathogenic *p63* mutations in seven allelic diseaseas.

Mutation	Exon	Domain	Isoform		Syndrome	Reference
p.N6H	3'	TA2	ΔN	α, β, γ	ADULT	(23)
p.R58C	3	TA	TA	α, β, γ	SHFM	(42)
p.G76W	4	-	ΔN, TA	α, β, γ	LMS	(12)
p.S90W	4	-	ΔN, TA	α, β, γ	LMS	*
3'ss intron 4	i4/e5	-	ΔN, TA	α, β, γ	SHFM	(6)
p.G134D	4	-	ΔN, TA	α, β, γ	LMS	*
p.G134D	4	-	ΔN, TA	α, β, γ	ADULT	(26)
p.L162P	5	DBD	ΔN, TA	α, β, γ	EEC	(11)
p.Y163C	5	DBD	ΔN, TA	α, β, γ	EEC	(12)
p.Y192C/D	5	DBD	ΔN, TA	α, β, γ	EEC	*
p.K193E	5	DBD	ΔN, TA	α, β, γ	SHFM	(6)
p.K194E	5	DBD	ΔN, TA	α, β, γ	SHFM	(5)
p.V202M	5	DBD	ΔN, TA	α, β, γ	EEC	(12,59)
p.R204L/Q/W	6	DBD	ΔN, TA	α, β, γ	EEC	(4,6,11,12,60,61)
p.H208Y	6	DBD	ΔN, TA	α, β, γ	EEC	(11)
p.R227Q	6	DBD	ΔN, TA	α, β, γ	EEC	(11,12)
p.C269Y	7	DBD	ΔN, TA	α, β, γ	EEC	(6)
p.S272N	7	DBD	ΔN, TA	α, β, γ	EEC	(4)
p.C273Y	7	DBD	ΔN, TA	α, β, γ	EEC	(6)
p.R279C/H/Q	7	DBD	ΔN, TA	α, β, γ	EEC	(4,5,12,31,60-63)
p.R279H	7	DBD	ΔN, TA	α, β, γ	RHS	(30)
p.R280C/H/S	7	DBD	ΔN, TA	α, β, γ	EEC	(6,12,29,51)
p.R280C/H	7	DBD	ΔN, TA	α, β, γ	SHFM	(5)
p.R298G/Q	8	DBD	ΔN, TA	α, β, γ	ADULT	(20,22,24,25)
p.R304P/Q/W	8	DBD	ΔN, TA	α, β, γ	EEC	(4-6,31,61,64,65)
p.C306Y/R	8	DBD	ΔN, TA	α, β, γ	EEC	(4,66)
p.C308S/Y	8	DBD	ΔN, TA	α, β, γ	EEC	(6)
p.P309S	8	DBD	ΔN, TA	α, β, γ	EEC	(6)
p.D312G/H/N	8	DBD	ΔN, TA	α, β, γ	EEC	(6,67)
p.R313G	8	DBD	ΔN, TA	α, β, γ	EEC	*
p.R313G	8	DBD	ΔN, TA	α, β, γ	NSCL	(8)
p.A315E	8	DBD	ΔN, TA	α, β, γ	EEC	*
3'ss intron 10	i10/e11	-	ΔN, TA	α, β, γ	AEC	(29)
p.I510T	13	SAM	ΔN, TA	α	RHS	(27)
p.I510T	13	SAM	ΔN, TA	α	AEC	(27,38)
p.L514F/S/V	13	SAM	ΔN, TA	α	AEC	(7,35)
p.G518V	13	SAM	ΔN, TA	α	AEC	*
p.C522G/W	13	SAM	ΔN, TA	α	AEC	(7)
c.1572insA	13	SAM	ΔN, TA	α	EEC	(4)
c.1576delTT	13	SAM	ΔN, TA	α	LMS	(12)
p.G530V	13	SAM	ΔN, TA	α	AEC	(7)
p.T533P	13	SAM	ΔN, TA	α	AEC	(7)
p.534insPhe	13	SAM	ΔN, TA	α	AEC	(33)
p.Q536L	13	SAM	ΔN, TA	α	AEC	(7)
p.I537T	13	SAM	ΔN, TA	α	AEC	(7,12)
p.S541F	13	SAM	ΔN, TA	α	AEC	*
p.S541P/Y	13	SAM	ΔN, TA	α	RHS	(32,36)
p.R555P	14	SAM	ΔN, TA	α, β	AEC	(35)
p.I558T	14	SAM	ΔN, TA	α, β	AEC	*
p.L563P	14	SAM	ΔN, TA	α, β	EEC	(11)
c.1709delA	14	TI	ΔN, TA	α, β	RHS	(30)
c.1721delC	14	TI	ΔN, TA	α, β	RHS	(11,37)
c.1742delC	14	TI	ΔN, TA	α, β	AEC	(12)
c.1743delAA	14	TI	ΔN, TA	α, β	LMS	(6)
c.1787delG	14	TI	ΔN, TA	α, β	RHS	(25)
c.1859delA	14	TI	ΔN, TA	α, β	RHS	(31)
c.1859delA	14	TI	ΔN, TA	α, β	AEC	*
p.K632X	14	TI	ΔN, TA	α, β	LMS	(11)
p.Q634X	14	TI	ΔN, TA	α, β	SHFM	(6)
p.E639X	14	TI	ΔN, TA	α, β	SHFM	(12)

* Authors' unpublished data

One other point mutation (p.S90W) is also located between the TA domain and DBD. Other LMS mutations are reported in the C-terminus: a TT deletion in the exon 13 and an AA deletion in exon 14 (6,12). These will affect only the p63α protein isoforms, where they are predicted to cause a frame shift and a premature stop codon. Also a stop mutation in the transcription factor inhibitory domain (TI) (p.K632X) has been identified in a sporadic LMS patient (11). The latter mutation is predicted to impair the suppressive effect of the TI domain towards the TA domain, thus increasing the transactivation activity (16).

ADULT syndrome

ADULT syndrome phenotype is most similar to LMS syndrome, although clear differences can be seen when observing larger families or patient populations. The main difference is the absence of orofacial clefting and the presence of hair and skin defects in the ADULT syndrome (Fig. 1). Teeth, skin and nail defects are constantly present in ADULT syndrome (100, 93 and 100%, respectively), but only rarely in LMS. Hair (53%) and lacrimal duct defects (67%) are observed in ADULT patients more frequently than in LMS. Freckling has been reported, but cannot be considered to be a differentiating feature of this syndrome (20).

By today, four ADULT syndrome families and three sporadic cases have been reported (20-26). All the families and one of the sporadic cases have a point mutation in exon 8, changing p.R298 in the DNA binding domain into either a glutamine or a glycine (Table 1). While EEC syndrome mutations in the DNA binding domain impair the binding of p63 protein to DNA (4), arginine p.298 is not located close to the DNA-binding interface, and mutation of this arginine does not affect DNA binding (24). Instead, earlier studies have shown a gain-of-function effect for the mutated ΔNp63γ isoform, which usually does not have a transactivation activity in assays using an optimized p53-responsive element (20,24). Two other mutations are located in the N-terminus: p.N6H mutation affects only the ΔN-isoforms and in another isolated patient a missense mutation p.G134D (In the article incorrect amino acid annotation, erratum pending Ref. Slavotinek et al. 2005(26)(19)) is located just front of the DBD in exon 4 (23,26).

AEC syndrome

The AEC syndrome phenotype differs from the other conditions mainly by the severity of the skin phenotype, the occurrence of an eyelid fusion at birth and the absence of limb malformations (Fig. 1). Approximately 80% of the patients have severe skin erosion at birth, which usually will recover in the first years of the life. The eyelid fusion, also called ankyloblepharon, is present in about 45% of AEC patients, but only rarely in other p63-associated conditions. The other ED symptoms, such as nail and teeth defects are present in more than 80% of patients, and hair defects and/or alopecia are almost constant features (94%). Lacrimal duct obstruction is seen in 50% of patients, whereas mammary gland hypoplasia and hypohydrosis occur occasionally (both 13%). Interestingly, almost 40% of patients have hearing impairment and genito-urinary defects. Cleft lip is present in 44% and cleft palate in about 80%. Limb malformations are almost absent. Ectrodactyly has never been reported, but 25% of patients has only mild syndactyly (11).

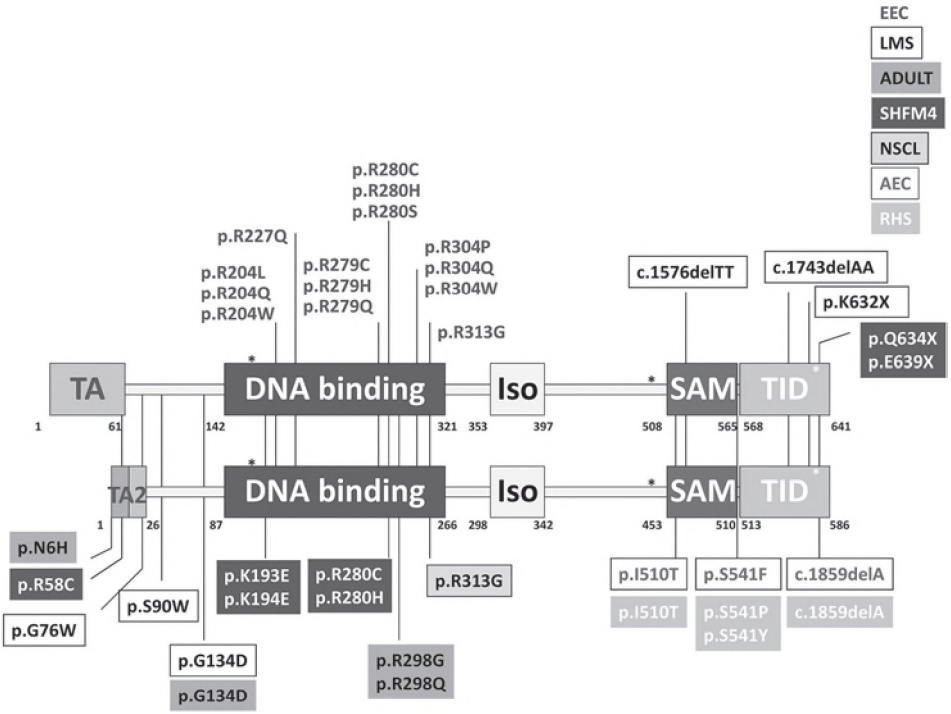

Figure 2. Pathogenic *p63* mutations cause at least five different syndromes and two non-syndromic conditions. Mutations causing different diseases are illustrated in different colours. Only mutations that are discussed in the text are indicated, an overview of all mutations is given in Table 1. EEC hotspot mutations are clustered in DNA binding domain, and RHS and AEC syndrome mutations in SAM and TI domains. Several mutations, such as p.R280, p.R313, p.I510, p.S541 and c.1850delA, can have a variable clinical outcome, probably due to genetic background effects. The black asterisks illustrate sites needed for upiquitination (p.K193, p.K194 and PY) and the white asterisk represents a sumoylation site (fKXD/E) (17-19).

Rapp-Hodgkin syndrome (RHS)

RHS mimics AEC very much (Fig. 1). The differences discussed earlier in several papers are the absence of ankyloblepharon in RHS and the more severe skin phenotype in AEC (11,27). Other ED symptoms, such as orofacial clefting and the near absence of limb malformations are similar to AEC. These two conditions could be considered as a single entity, since the ankyloblepharon is present only in about 45% of AEC syndrome patients, and therefore is not a discriminating factor. Although, the severity of the skin phenotype is obvious and more severe in AEC patients than in RHS patients, the strong overlap between AEC and RHS suggest, that they are variable manifestations of the same clinical entity (11,27).

AEC and Rapp Hodgkin syndrome mutations are located in the C-terminus of the p63 protein (Fig. 2). They are either point mutations in the SAM domain or deletions in the SAM or TI domains (Table 1) (7,11,12,27-38). The SAM domain is known to be involved in protein-protein interactions and therefore mutations in this domain are most probably hampering the binding to interacting proteins. One known interactor of p63 SAM domain is the Apobec1-binding protein 1 (ABBP1),

which is a member of RNA processing machinery and known to regulate the alternative splicing of the Fibroblast growth factor receptor 2 (FGFR2) towards the epithelial specific isoform. AEC mutations in the SAM domain abolish the binding to ABBP1, which most probably leads to changes in FGFR2 RNA splicing (39). Interestingly, gain-of-function mutation in FGFR2 gene have been reported in a number of craniosynostosis syndromes, which are also characterized by distal limb malformations (40). Recently, loss-of-function mutations in FGFR2 have been found in Lacrimo-auricolo-dento-digital syndrome (LADD), an ED syndrome characterized by dominant inheritance of limb defects in association with abnormal lacrima, ear cups and teeth (41). Interestingly, LADD shows marked overlap with EEC syndrome, and was earlier also presumed to be caused by mutations in the *p63* gene.

Non-syndromic p63 conditions

Split hand/foot malformation type 4 (SHFM4) is a "pure" limb malformation (ectrodactyly and syndactyly) condition, thus without orofacial clefting or ectodermal dysplasia. The non-syndromic SHFM4 is caused by several mutations, which are dispersed throughout the *p63* gene: a point mutation in the Transactivation domain (TA) (p.R58C), a splice-site mutation in front of exon 4 (3'ss intron 4), four missense mutations in the DNA binding domain (p.K193E, p.K194E, p.R280C, p.R280H), and two nonsense mutations in the TI-domain (p.Q634X, p.E639X) (Table 1) (5,6,42). It is still unclear how these widely dispersed mutations cause the limb defect. Interestingly, several SHFM4 mutations are reported to cause alteration in the p63 protein activation and stability: p.Q634X and p.E639X are known to disrupt the sumoylation site, and therefore increase the stability and transcriptional activity of the p63α isoform (19,43). Furthermore, amino acids p.K193 and p.K194 are required for ubiquitin conjugation by E3 ubiquitin ligase (Itch) and naturally occurring mutations in those amino acids cause more stable p63 protein (17). Possibly, SHFM is caused by altered protein degradation, even though different degradation routes are involved. Another divergent phenomenon is the p.R280C/H mutations, which are not only causative for SHFM4, but also for the syndromic EEC phenotype in other families. In such families, the phenotype is always consistent, either EEC or SHFM4. In SHFM4 families decreased penetrance is reported, for the p.R280 mutation, suggesting an effect of a nearby genetic modifier.

Recently, a non-syndromic orofacial clefting type was also linked to *p63* gene (8). The amino acid change p.R313G is the first mutation causing the Non-syndromic cleft lip (NSCL) phenotype in the *p63* gene (8). This mutation was also observed in a sporadic EEC syndrome phenotype (authors' unpublished data). Mutations and/or polymorphisms in 3 other genes, *IRF6, MSX1* and *PVRL,* are also associated with a syndromic and non-syndromic forms of orofacial clefting (44-50). In the study of Leoyklang et al (8) also other changes in the *p63* gene were found (p.N87N, p.S90L, p.L248L, p.H406H, p.D564H), but because the mutations did not change the amino acid sequence or were also found in one of the healthy parents or in the control population, they were not considered to be pathogenic changes. Nevertheless, since orofacial clefts are considered to have a multifactorial origin it is quite possible that these changes in the *p63* gene impose a risk factor for facial clefting type, similar as was reported for the *IRF6* gene.

Discussion

The mutation patterns in the *p63* gene that are associated with different *p63* clinical conditions display a clear genotype-phenotype association: especially in the EEC and AEC/RHS syndromes, where mutations are clustered in the DNA binding domain and in the SAM and TI domains, respectively. In about 250 patients we and others have described 74 different mutations causing five different syndromes and two different non-syndromic conditions. This still increasing number of patients allows the delineation of the phenotypic and genotypic pictures of different p63-associated disorders. In addition, evidence is accumulating that other genetic factors influence the final *p63* mutant phenotype.

Several examples show that the same mutation can lead to different clinical conditions. AEC and RHS syndromes share three mutations: p.I510, p.S541 and c.1859delA (27,28,31,32,34,36). Often the clinical distinction between RHS and AEC syndrome is solely based on the presence or absence of ankyloblepharon, which in fact is not the discriminating feature between these two conditions (11). Also LMS and ADULT syndrome seem to be caused by the same mutation p.G134D (authors' unpublished data) (26), as well as EEC syndrome and Non-syndromic cleft lip/palate due to the p.R313G mutation (authors' unpublished data) (8). Also several frameshift mutations are found, interestingly always with variable phenotype, thus causing EEC, LMS, AEC or RHS syndrome. The possible explanation may be a misdiagnosis in sporadic cases, because of the phenotypic variability, likely, however frameshift mutations are exceptionally sensitive to the effects of modifier genes. An example of family-dependent clinical phenotype is the arginine p.280 mutation, which can be mutated to cysteine or histidine and lead either to the non-syndromic SHFM4 (2 families) or more often to syndromic ectodermal dysplasia with SHFM and orofacial clefting (EEC) (9 families) (5,6,29,51). Surprisingly, the penetrance is reduced in SHFM families, but not in EEC families, which also indicates a modifier effect somewhere else in the genome.

The phenotypic variation between the p63-linked diseases is large, furthermore the phenotypic variation within one disease is also considerable. It is clear that variability within families may be ascribed to a combination of modifier genes, and stochastic processes. No such modifier genes have yet been identified for the p63 syndromes described in this review. However, modifiers have been identified for the phenotypic effects of *p53* gene mutations in human cancer, and at least one of these has also been shown to affect p63 protein levels and transcription (52,53). Other candidate modifier genes may be found in pathways that are known to affect specific phenotypes. For instance, genetic variation in the *IRF6* gene could be tested as a risk factor for cleft lip in p63 mutant families (48-50).

Furthermore, variation between families likely reflects differential downstream effects for each of these mutations. There are many possible ways in which this can be achieved, as the p63 protein is expressed in several isoforms, which are involved in different cellular functions during the embryonal development but also later on. The variation in the phenotypes caused by each of the five EEC hotspot mutations is also striking. The EEC hotspot mutation data, where clear phenotypic variation is seen between the mutations inside a single clinical syndrome, might reflect that different target genes are affected by each of the hotspot mutations. Whether this is so, needs to be examined in more detail in animal models or cellular systems. Clearly, these EEC mutations cannot just be simple loss-of-function or dominant negative mutations, but gain-of function mutations are likely involved, as was described for *p53* mutations in transgenic mice (54-56).

Finally, the finding that some mutations such as p.R280C/H have different phenotypes between families, but the same phenotype consistently within families suggest the influence of cis-acting polymorphisms, either in *p63* itself or within a genetically linked gene on chromosome 3q. Such functional polymorphisms have not yet been described, but here too, there are precedents for the existence of such polymorphisms in both *p53* and *p73* (57,58). Each of these possible mechanisms for phenotypic modulation will require functional hypotheses that can be verified in experimental systems, and ultimately be tested for their impact on the many families that are affected by these clinically often severe malformations.

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Pattern of *p63* mutations and their phenotypes -Update

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Abstract

Heterozygous mutations in the transcription factor gene *p63* cause at least six different syndromes with various combinations of ectodermal dysplasia, orofacial clefting and limb malformations. Here we will present an update of mutations in the *p63* gene together with a comprehensive overview of the associated clinical features in 227 patients. These data confirm the previously recognized genotype-phenotype associations. Moreover, we report that there is a large degree of clinical variability in each of the p63-associated disorders. This is illustrated by the different phenotypes that are seen for the five hotspot mutations that explain almost 90% of all EEC syndrome patients.

Introduction

Ectodermal dysplasias (EDs) constitute a large and complex group of developmental syndromes, comprising more than 170 different clinical conditions. The combined incidence of ED is approximately seven in 10,000 births. Causative genes for EDs have been identified in approximately one fifth of EDs. One group of EDs is associated with orofacial clefting and split-hand/foot malformation (SHFM). Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC, OMIM 604292) is the prototype of these syndromes, which is caused by heterozygous mutations in the *p63* gene. A number of EEC-like syndromes has been described, five of which are also caused by mutations in the transcription factor gene *p63*: Ankyloblepharon-ectodermal defects-cleft lip/palate (AEC, OMIM 106260), Limb mammary syndrome (LMS, OMIM 603543), Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, 103285), Rapp-Hodgkin syndrome (RHS, OMIM 129400) and non-syndromic Split hand/foot malformation (SHFM4, OMIM 605289).

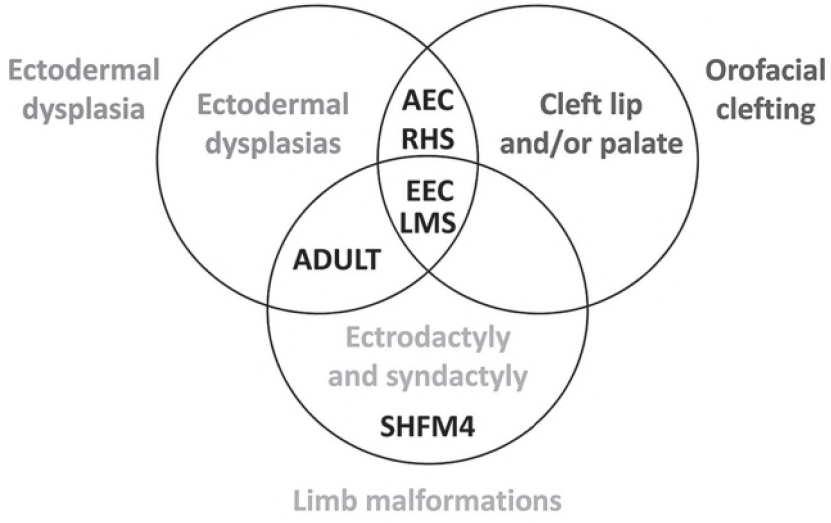


Figure 1. Ectodermal dysplasia (ED) combined with orofacial clefting and limb malformations are the three hallmarks of p63 syndrome family. EEC, the prototype of these syndromes combines all three main symptoms, whereas the others lack mainly one of the hallmarks. LMS falls into a borderline expressing less ectodermal signs than EEC. Ectodermal dysplasia and orofacial clefting are main symptoms in AEC and RHS, whereas limb malformations replace the orofacial clefting symptoms in ADULT syndrome.

These syndromes share at least one of the three main phenotypic hallmarks with EEC syndrome (Fig. 1). Previously, syndrome-specific mutation patterns have been identified upon analysis of *p63* mutations in 78 families, comprising five different syndromes (RHS was not elucidated). Here we will provide an update of *p63* mutation patterns by providing detailed clinical overviews of 126 unrelated families in which a causative *p63* mutation has been identified. The combined data confirm the genotype-phenotype associations that have been previously uncovered. In addition, it is becoming clear that phenotypes differ for the five hotspot mutations that account for the large majority of patients with EEC syndrome.

EEC - Prototype of p63 syndromes

EEC syndrome comprises limb malformations, ED and orofacial clefting. Representative limb malformations are ectrodactyly and syndactyly. ED is seen as light colored, sparse hair and absence of eyelashes, eyebrows and alopecia can be observed. Skin is thin and dry, sometimes resembling dermatitis. Nails are usually dystrophic and have pits. Also dental changes are reported such as hypodontia or anodontia and teeth can be prone to caries, because of enamel defect and salivary gland malfunction. Orofacial clefting is frequent, and facial maxillary and mild malar hypoplasia can be present. Hearing loss is uncommon. Choanal atresia is rare. Lacrimal duct stenosis contributes to keratitis, which is common in EEC patients, sometimes associated with photophobia. Genito-urinary malformations are also part of the EEC: sometimes external and internal genitalia are abnormal, but more often malformation has affected kidney, ureters or bladder. Mental retardation is rare in EEC, and probably as common as in the general population. These are the main symptoms that can be generally observed in EEC syndrome. However, there is a large degree of clinical variability, as we will demonstrate below.

EEC syndrome has been localized to three different chromosomes, although only one causative gene has been found (1). EEC1 has been mapped to chromosome location 7q21 both by the identification of chromosomal abnormalities, translocations and deletions in syndromic SHFM patients and by linkage analysis in a single large EEC1 family (2,3). However the chromosome 7 phenotype differs from classical EEC in that the patients have fewer ectodermal defects and present sensorineural deafness and ear malformations. EEC2 denotes linkage to chromosome 19 in one large EEC family (4,5). EEC3 on 3q27, is characterized by mutations of the *p63* gene (1). Interestingly, a common *p63* mutation, typical for EEC syndrome (p.R227Q) was also found in the EEC2 family, indicating that the linkage to chromosome 19 was a spurious finding. Indeed, *p63* mutations have been demonstrated in 98% of patients with a classical EEC phenotype [(6) and our own unpublished data]. Mutations in p63-derived EEC syndrome (EEC3) have been reported previously in 152 cases. These comprise 26 families and 60 sporadic cases (6-18). All mutations are point mutations clustered in the DNA binding domain, except for one frameshift mutation, which is located in the Sterile alpha motif (SAM) domain (1). The most frequently mutated amino acids residues are p.R204, p.R227, p.R279, p.R280 and p.R304, which cover 86.8% of all EEC syndrome cases. These mutation hotspots are the same as previously reported (19) and mimics p53 mutation hotspots entirely, except for the p.R227 residue. All of the hotspot missense mutations are C to T transitions at CpG islands.

A compilation of the manifestations of the 152 p63-associated EEC patients provides a more complete picture of the main characteristics observed in patients, the mutation patterns and mutation-specific phenotypes (Table 1). Ectodermal tissues, such as hair, teeth, nails and lacrimal

ducts are affected in about one half of the EEC patients, whereas abnormal skin was reported only in one third of the patients. Clefting of lip and palate is present in approximately 40% of the patients. Isolated cleft lip or cleft palate was observed only in rare instances (two and four cases, respectively). Limb malformations are a main component of the syndrome with ectrodactyly in two thirds of EEC cases and syndactyly less frequent in about 40% of patients. Minor characteristics in EEC are mammary gland and/or nipple hypoplasia, which are present in approximately 15% of the cases. Urinary and kidney problems may cause serious morbidity, and these were reported in 15% (Table 1).

EEC hotspot mutations demonstrate mutation-specific phenotype patterns

Each of the five EEC hotspot mutations was found in 24-31 patients. Remarkable phenotypic differences can be observed for these different mutations. Amino acid p.R204 is located in the beginning of the DNA binding domain and was mutated from arginine to tryptophan, glutamine or leucine. All patients were clinically diagnosed as EEC except for one patient with p.R204Q who was considered to have the Limb mammary syndrome, apparently because of mammary gland hypoplasia (Table 1). The p.R204 mutation phenotype is very similar to the overall EEC syndrome phenotype. Exceptions are a lower frequency of hypohidrosis and orofacial clefting (Fig. 2B).

Amino acid p.R227 is known to be mutated from arginine to glutamine, and was observed in 25 EEC patients and one with LMS. Mutations of this amino acid are rarely associated with orofacial clefting. Only two patients had clefts and these involved only the palate. This is striking since approximately 40% of all EEC patients present clefting, almost always both cleft lip and cleft palate. Patients with p.R227 mutations also have fewer limb defects. Ectrodactyly was present in 40% (11/28) versus 68% for the EEC group as a whole. Syndactyly was never reported. On the other hand, kidney and urinary problems are quite common in p.R227 mutation carriers occurring in 11/28 patients (40% vs. 12% for EEC patients with other mutations). Hypohidrosis is also common, whereas no hearing impairment is detected (Fig. 2C). These characteristics indicate that this amino acid p.R227 differs importantly from the other hotspot mutations in terms of function. Interestingly, arginine p.227 is the only *p63* mutation hotspot that does not have a homologous *p53* mutation hotspot as well (1).

At the end of the DNA binding domain there are three EEC mutation hotspots in close proximity: p.R279, p.R280 and p.R304. Each of these has its own typical phenotype pattern. p.R279 can mutate from arginine to histidine, cysteine or glutamine. All 24 mutations occurred in EEC syndrome patients, except for one patient who was diagnosed as Rapp-Hodgkin syndrome (20). Interestingly this is the only EEC mutation, which can give rise to ankyloblepharon, as reported in two cases, indicating limited overlap with AEC syndrome (15). Other specific characteristics for the p.R279 mutation are a high incidence of ectrodactyly (18/23) and perhaps hypohidrosis (4/23) (Fig. 2D). The next amino acid p.R280 is known to be mutated from arginine to cysteine, histidine or serine. Symptoms mimic the common EEC pattern, with frequent skin signs and frequent syndactyly (17/27). Interestingly, no sweating, hearing or kidney problems were reported in patients with a p.R280 mutation (Fig. 2E). The last EEC hotspot mutation is at amino acid p.R304, which can be mutated to tryptophan, glutamine or proline. The most striking finding in patients with this mutation is a very high percentage of orofacial clefting (22/27 patients), frequent occurrence of syndactyly and of hearing impairment (Fig. 2F).

Table 1. Phenotypic characteristics of five human ectodermal dysplasias associated with p63 mutations.

	Families	Patients	Hair	Lacrimal duct	Nail	Skin	Teeth	Cleft Lip	Cleft Palate	Ectrodactyly	Syndactyly	Hypo-hydrosis	Mammary gland/nipple hypoplasia	Ankylo-blepharon	Urinary/ Kidney	Hearing impairment	Reference
EEC																	
p.L162P	**1**	**2**	2	0	1	0	2	1	1	2	2	0	0	0	0	0	#
p.Y163C	**1**	**1**	1	1	1	1	1	0	1	0	1	1	1	0	0	0	(19)
p.V202M	**2**	**2**	1	1	1	1	1	1	1	2	0	0	0	0	0	0	(16,19)
p.R204L/Q/W	**16**	**26***	16	13	15	9	11	7	7	17	11	1	3	0	4	2	(1,6,13,15,19)
p.H208Y	**1**	**1**	1	1	1	1	1	0	0	0	1	0	0	0	0	0	#
p.R227Q	**6**	**25**	17	19	14	6	18	0	1	11	0	8	7	0	8	0	(19)
p.C269Y	**1**	**2**	2	1	1	0	0	2	2	2	2	0	0	0	0	0	(6)
p.S272N	**1**	**1**	1	1	0	0	1	1	1	1	1	0	1	0	0	1	(1)
p.C273Y	**1**	**1**	1	0	0	0	0	1	1	1	1	0	0	0	0	0	(19)
p.R279C/H/Q	**16**	**23****	11	13	9	6	11	11	11	18	7	4	4	2	2	1	(1,8,11,13-15,19)
p.R280C/H/S	**9**	**31**	20	8	10	12	13	8	8	23	17	0	2	0	0	0	(6,9,17,19)
p.R304P/Q/W	**21**	**27**	16	20	16	7	12	22	22	20	16	1	1	0	7	5	(1,6,7,10,13,14)
p.C306R/Y	**2**	**2**	2	1	2	2	1	2	1	2	2	1	1	0	1	0	(1,18)
p.C308S/Y	**2**	**2**	2	2	2	2	1	0	0	0	2	0	1	0	1	0	(6)
p.P309S	**1**	**1**	1	1	1	1	1	0	0	1	0	0	0	0	0	0	(6)
p.D312G/H/N	**3**	**3**	3	3	2	3	3	3	3	3	2	1	0	0	0	1	(6,12)
c.1572insA	**1**	**1**	0	1	0	0	1	1	1	1	0	0	1	0	0	1	(1)
p.L563P	**1**	**1**	1	0	1	0	1	0	0	0	1	0	0	0	0	0	#
Total	**86**	**152**	**98**	**86**	**77**	**51**	**79**	**60**	**61**	**104**	**66**	**17**	**22**	**2**	**23**	**11**	
Percentage			**66**	**57**	**52**	**34**	**53**	**39**	**40**	**68**	**43**	**11**	**14**	**1**	**15**	**7**	
* ED of two patients not described in details, not taking to account in %. ** ED of one patient not described in detailes, not taking to account in %. # Authors unpublished data.																	
ADULT																	
p.N6H	**1**	**1**	0	0	1	0	1	0	0	0	1	0	1	0	0	0	(27)
p.R298G/Q	**4**	**14**	8	10	14	14	14	0	0	9	8	1	11	0	0	0	(22-26)
Total	**5**	**15**	**8**	**10**	**15**	**14**	**15**	**0**	**0**	**9**	**9**	**1**	**12**	**0**	**0**	**0**	
Percentage			**53**	**67**	**100**	**93**	**100**	**0**	**0**	**60**	**60**	**7**	**80**	**0**	**0**	**0**	

Mammary gland hypoplasia is common in both LMS and ADULT syndrome

LMS was the first p63 syndrome linked to chromosome region 3q27 (6,19,21). The LMS phenotype comprises malformations of the hands and/or feet and hypoplastic nipples and/or mammary glands. Ectodermal defects are much less prominent than in EEC syndrome and there are no hair and skin anomalies. Patients from six families have been described with this syndrome, four of these are sporadic cases making it difficult to establish whether they are real LMS patients or examples of variable expressivity of other p63 syndromes. Indeed, two cases diagnosed as LMS, have mutations that are typical of EEC syndrome: p.R204Q and p.R227Q. Nonetheless, the only large LMS family reported to date is clearly different from EEC (21), and this family and two further LMS patients have mutations that have never been observed in EEC. In contrast to EEC syndrome, LMS patients rarely have any hair and skin involvement and if clefting is present, it is always limited to the palate. Mammary gland and/or nipple hypoplasia or aplasia is more frequent in LMS than in EEC (100% and 29% of LMS cases; Table 1). Hypohidrosis is relatively frequent (29%).

ADULT syndrome is clinically very similar to LMS, because mammary hypoplasia plays a main role in both syndromes. Nevertheless, there are clear differences. First of all, orofacial clefting has not been observed in ADULT patients, whereas nail, skin and teeth are affected in almost every case. Hypohidrosis is present in about 30% of LMS patients and was reported in ADULT syndrome in only 7% (1/14). (Table 1).

ADULT syndrome is usually caused by "an ADULT syndrome hotspot" point mutation affecting amino acid p.R298 in exon 8 at the end of the DNA binding domain. There are five unrelated families with p.R298Q/G mutation each expressing more or less similar ADULT syndrome characteristics (22-26). The first ADULT syndrome family was described to have intensive freckling (22,23). Other ADULT syndrome families did not show increased freckling and therefore freckling does not seem to be a component of this syndrome. A further ADULT mutation p.N6H was reported in the alternative N-terminus (27). LMS and ADULT syndromes overlap, and differentiating them can be difficult, especially in sporadic cases (26).

AEC and Rapp-Hodgkin syndrome

Two other syndromes belong to the p63 syndrome family. Hay-Wells syndrome, also known as Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC), which was first reported by Hay and Wells (28). Its main symptoms are ankyloblepharon (fusion of the eyelids), ectodermal defects and cleft lip and palate. About 75% of patients have severe skin erosions at birth, with some AEC patients reported to have up to 70% denuded skin which resembles a second-degree burn (29). Normal neonatal skin is slowly recovered. By 4-5 years age erosions have usually disappeared except for the head and auricular region. Alopecia is also often linked to Hay-Wells syndrome as are absence of eyelashes and eyebrows. Clefting occurs approximately in 80% of AEC patients, mostly cleft palate or cleft lip and palate. It should be noted that the denominative ankyloblepharon occurs only in 44% of AEC cases. Hearing loss has been reported in about 40% of the patients. AEC patients have nail and teeth defects in about 75-80% of cases. About half of the patients have lacrimal duct atresia. Sweating abnormalities and mammary gland and/or nipple hypoplasias are rarely observed.

Table 1. *(continues)*

	Families	Patients	Hair	Lacrimal duct	Nail	Skin	Teeth	Cleft Lip	Cleft Palate	Ectrodactyly	Syndactyly	Hypo-hydrosis	Mammary gland/nipple hypoplasia	Ankylo-blepharon	Urinary/ Kidney	Hearing impairment	Reference
LMS																	
p.G76W	**1**	**27**	0 of 21	7 of 15	7 of 21	0 of 21	3 of 21	-	6 of 22	21 of 27	-	7 of 21	27 of 27	-	-	-	(19,21)
p.R204Q*	**1**	**1**	0	1	1	0	1	0	0	0	1	0	1	0	0	0	#
p.R227Q*	**1**	**3**	3	3	3	3	2	0	0	0	0	0	3	0	3	1	#
c.1576delTT	**1**	**1**	0	0	0	0	1	0	1	1	0	0	1	0	0	0	(19)
p.K632X	**1**	**1**	0	1	1	0	1	1	1	1	1	1	1	0	0	0	#
c.1743delAA	**1**	**1**	1	1	1	1	0	0	1	1	1	0	1	0	0	0	(6)
Total	**6**	**34**	**4**	**13**	**13**	**4**	**8**	**1**	**9**	**24**	**3**	**8**	**34**	**0**	**3**	**14**	
Percentage			**14**	**59**	**46**	**14**	**29**	**3**	**31**	**71**	**9**	**29**	**100**	**0**	**10**	**0**	
RHS																	
p.R279H*	**1**	**1**	1	1	1	1	1	0	0	0	1	1	0	0	1	0	(20)
p.I510T (+ p.P472T)	**1**	**1**	1	1	1	1	0	0	1	0	1	0	0	0	0	0	(32)
p.S541P/Y	**2**	**2**	2	2	2	1	2	2	2	0	1	0	1	0	0	1	(31,39)
p.1709delA	**1**	**1**	1	0	1	0	1	0	1	0	0	1	0	0	1	0	(20)
p.1721delC	**1**	**1**	1	0	1	0	1	0	0	0	0	1	0	0	0	0	#
p.1787delG	**1**	**1**	1	1	0	1	1	0	1	0	0	0	0	0	0	1	(38)
p.1859delA	**1**	**3**	3	3	0	0	3	1	2	0	0	0	0	1	0	0	(14)
Total	**8**	**10**	**10**	**8**	**6**	**4**	**9**	**3**	**7**	**0**	**3**	**3**	**1**	**1**	**2**	**2**	
Percentage			**100**	**80**	**60**	**40**	**90**	**30**	**70**	**0**	**30**	**30**	**10**	**10**	**20**	**20**	
AEC																	
3' ss i10/e 11	**1**	**3**	3	1	3	3	3	0	1	0	0	0	0	0	3	0	(9)
p.I510T	**1**	**1**	1	1	1	1	1	0	0	0	1	0	0	1	0	0	(32,37)
p.L514F/V	**2**	**2**	2	2	2	2	1	1	2	0	0	0	0	2	1	2	(33)
p.C522G/W	**2**	**2**	1	0	0	2	1	2	2	0	1	0	1	0	1	1	(33)
p.G530V	**1**	**1**	1	0	0	1	1	1	1	0	1	0	0	0	0	1	(33)
p.T533P	**1**	**2**	2	2	2	0	2	0	2	0	1	0	1	2	0	0	(33)
p.534insPhe	**1**	**1**	1	0	1	1	0	1	1	0	0	0	0	0	0	0	(29)
p.Q536L	**1**	**1**	1	0	1	0	1	0	1	0	0	0	0	0	0	0	(33)
p.I537T	**2**	**2**	2	1	2	2	2	1	2	0	0	1	0	2	1	1	(19,33)
c.1742delC	**1**	**1**	1	1	1	1	1	1	1	0	0	1	0	0	0	1	(19)
Total	**13**	**16**	**15**	**8**	**13**	**13**	**13**	**7**	**13**	**0**	**4**	**2**	**2**	**7**	**6**	**6**	
Percentage			**94**	**50**	**81**	**81**	**81**	**44**	**81**	**0**	**25**	**13**	**13**	**44**	**38**	**38**	

*Typical EEC syndrome mutation, # Authors unpublished data.

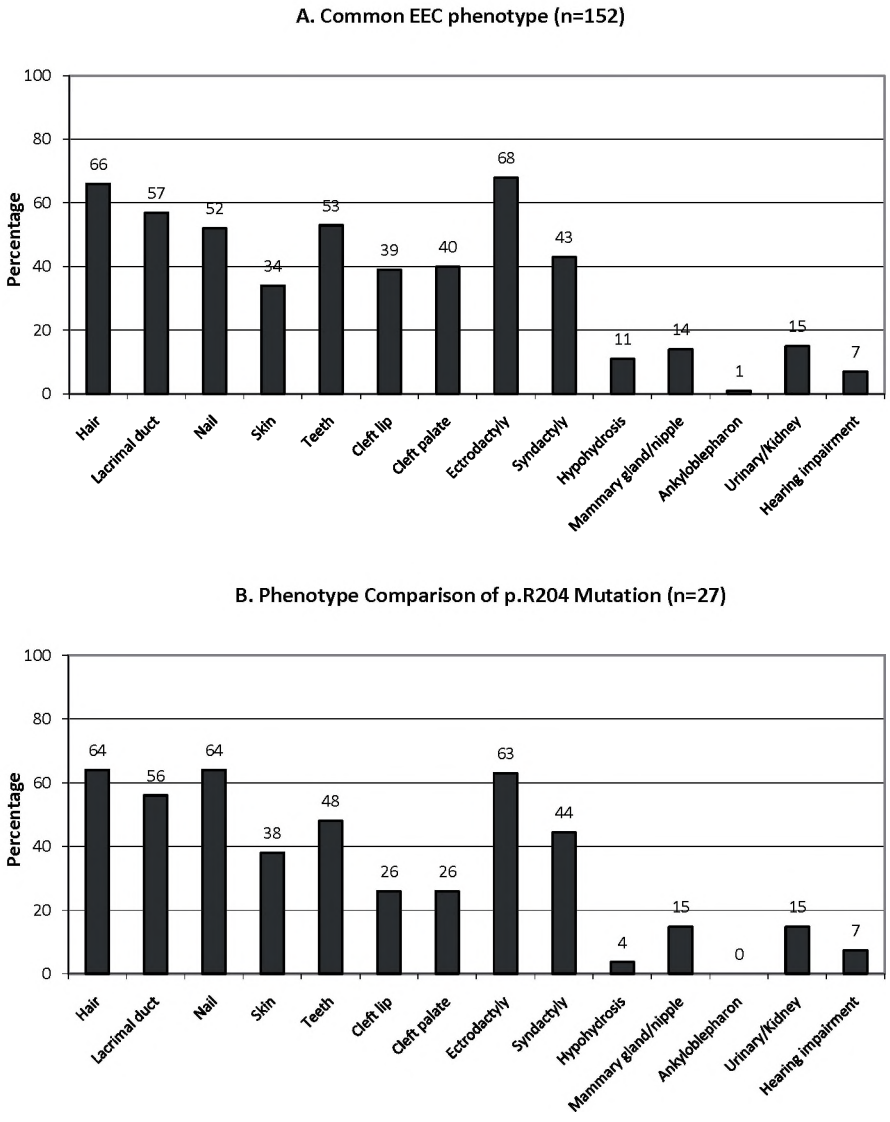


Figure 2. EEC hotspot mutations allow the specific phenotype delineation. Striking differences are especially between orofacial clefting and limb malformations.

One striking difference to the other p63 syndromes is the absence of limb malformations, which is almost complete, a few patients having only mild syndactyly. The clinical picture of AEC syndrome patients is thus unmistakably different from other p63-derived syndromes, with the exception of RHS (see below).

Rapp-Hodgkin Syndrome (RHS) was only recently shown to be a member of the p63 syndrome family. It was first described in 1968 by Rapp and Hodgkin (30). Kantaputra et al (31) linked this syndrome to defects in the *p63* gene. The ectodermal dysplasia in Rapp-Hodgkin syndrome manifests as sparse, fine hair with progressive alopecia, nail defects, hypodontia, lacrimal duct atresia and dry skin with a decreased number of sweat pores causing hypohidrosis.

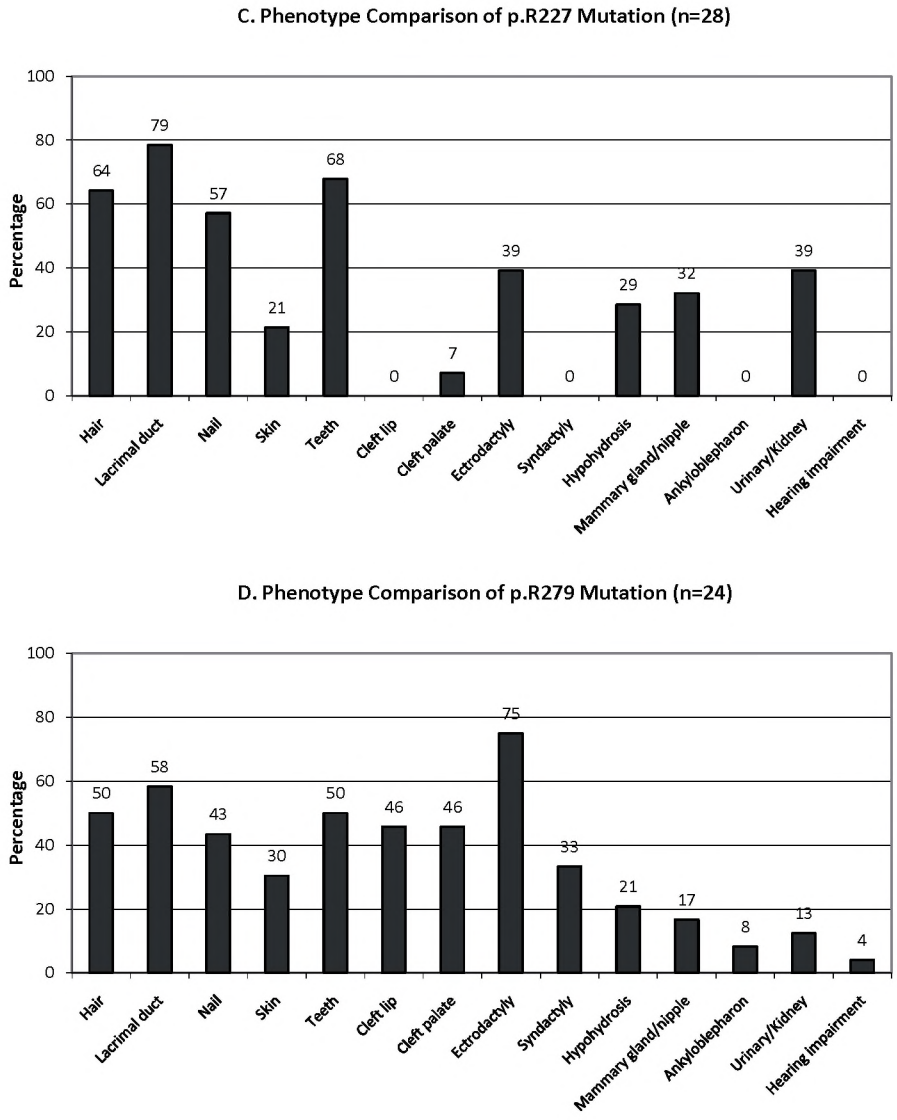
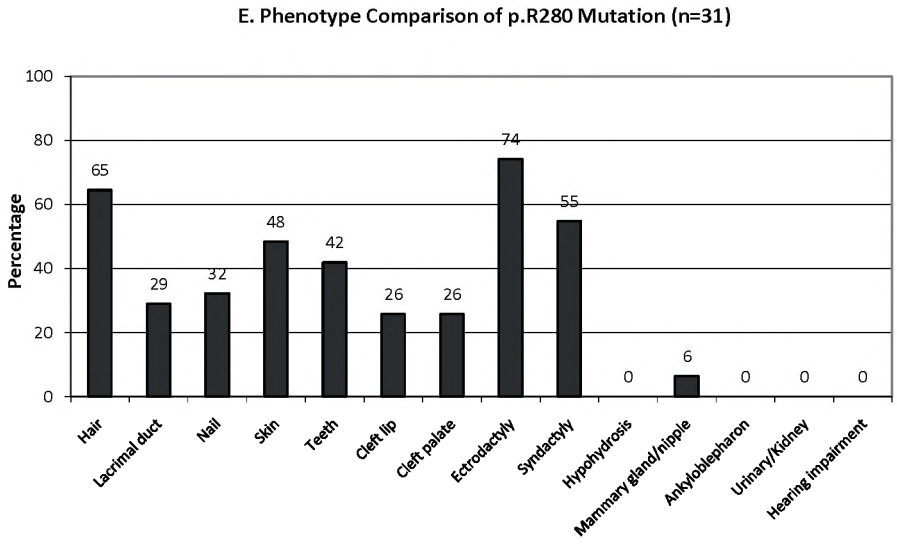


Figure 2. EEC hotspot mutations allow the specific phenotype delineation. Striking differences are especially between orofacial clefting and limb malformations.

Milder skin symptoms in RHS are probably the main clinical distinction between Hay-Wells and Rapp-Hodgkin syndrome. Presence of ankyloblepharon supports a diagnosis of AEC. However, since more than half of all AEC patients have no ankyloblepharon, a lack of this anomaly should not be considered as distinguishing for RHS (14,32). Syndactyly has been reported in 30% of the RHS cases, whereas among AEC patients it has been described only once (1/16 or 6%) (33). In both syndromes clefting in lip and/or palate is equally frequent. Genito-urinary defects are more common in RHS and AEC than in EEC syndrome, affecting every fourth patient. Also hearing impairment is more common in RHS and AEC than it is in EEC syndrome, 20, 38 and 7% of patients being affected, respectively.



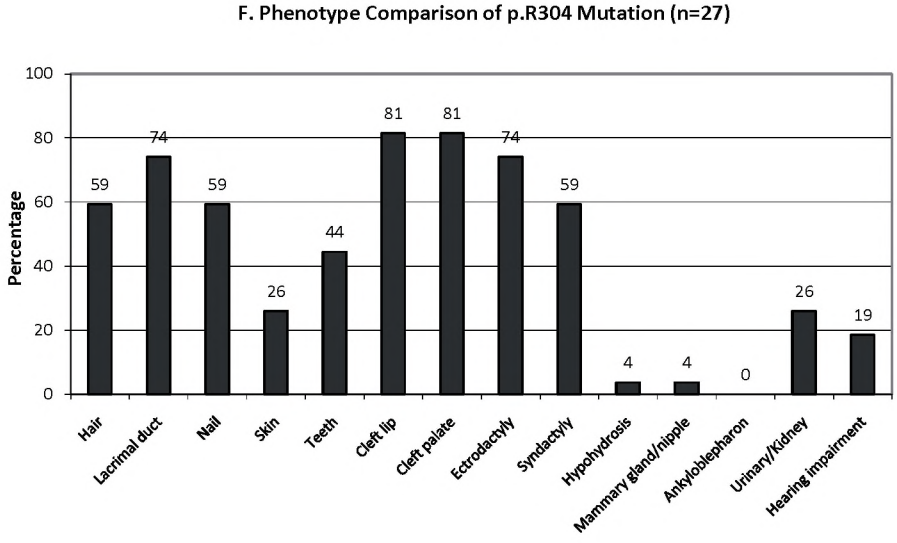


Figure 2. EEC hotspot mutations allow the specific phenotype delineation. Striking differences are especially between orofacial clefting and limb malformations.

SHFM

Like EEC syndrome, non-syndromic Split hand/foot malformation (SHFM) has been linked to several chromosomal loci in the human genome. In our experience, about 10% of SHFM patients have a *p63* mutation, referred to as SHFM4 (34). To date eight SHFM mutations have been described, which are dispersed along the *p63* gene. Various types of mutations are seen: splice-site mutation (3'ss intron 4), missense mutations (p.R58C, p.K193E, p.K194E, p.R280C/H) and stop-mutations (p.Q634X, p.Q639X) (6,34,35).

Genotype-phenotype associations

Table 1 summarizes the clinical data on 227 patients with a causative *p63* mutation, allowing the delineation of the typical phenotypic appearance of each syndrome. Moreover, especially in EEC and AEC syndromes mutations cluster in specific protein domains, providing molecular support for their phenotypic distinction. EEC mutations are missense and are dispersed along the DNA binding domain with just five hotspots involving some >80% of patients. There are two mutations outside the DNA binding domain, a point mutation (p.L563P) at the end of Sterile alfa motif (SAM) domain and a frameshift mutation (c.1572insA) at the beginning of the SAM domain. It has been predicted, that EEC mutations disturb the binding of the p63 protein to DNA, which leads to a loss of transactivation (1). In contrast, missense mutations in the SAM domain are mainly found in AEC (12/16), although a splice-site mutation at the intron 10/exon 11 boundary and two frameshift mutations were also detected. A SAM domain is mainly found in developmental genes and is thought to participate in protein-protein interactions (36). Very likely AEC mutations in the SAM domain inhibit these specific protein-protein interactions.

Clinical variability: One mutation can lead to two syndromes

Mutations in AEC and RHS are dispersed along SAM and Transactivation inhibitory (TI) domains, and the same amino acid mutations have been reported in both syndromes. For instance amino acid residue p.I510 located at the beginning of the SAM domain was found to be mutated in two sporadic cases (32). The AEC patient had mild ankyloblepharon, severe erythrematous plaque on the scalp, which improved by age of four months, sparse hair which later became wiry, coarse and curly, sparse eyebrows and eyelashes, hypodontia and decreased enamel, bilateral choanal atresia and absence of lacrimal puncta. He had mild cutaneous syndactyly and dysplastic nails, mild microretrognathia and asymmetric ears, intact palate and no history of heat intolerance (37). The other patient presented with persistent erythematous lesions on the scalp, back and genitalia, cleft palate, lacrimal duct atresia, syndactyly, nail dysplasia and hair defects and anterior displaced anus. Although their phenotypes are quite similar, they were diagnosed as having AEC and RHS, respectively. This distinction appears to have been based on the presence of ankyloblepharon in the first patient. Both patients were carrying the same mutation p.I510T, although the RHS patient also carries a p63 polymorphism p.P472T that was also in his healthy mother. It is presently unknown whether this rare p.P472T polymorphism has any effect on the phenotype.

A second mutated amino acid residue p.S541, has also been reported to cause either AEC or RHS. The p.S541P/Y mutation was seen in an RHS family and p.S541F in an AEC syndrome patient (31,32,38,39). A third example is c.1859delA occurred in a family where one individual had ankyloblepharon, cleft palate and ectodermal dysplasia, and two other affected members only ectodermal dysplasia with or without clefting (14). Overall the data suggest that AEC and RHS are one single syndrome with clinical variability.

However, there is a good example of one *p63* mutation causing two distinct syndromes. p.R280 mutations to cysteine and histidine were described in EEC syndrome and in a large SHFM family (6,9,17,34). So far, when arginine mutates to serine, it causes only EEC (6). The phenotype is fully consistent within families carrying each of these p.R280 mutations. Either there is full-blown EEC syndrome or isolated SHFM. In the latter families, non-penetrance is observed (34), which

is never seen in EEC syndrome patients. This strikingly consistent phenotype within families suggests the involvement of a modifier allele close to the *p63* gene on chromosome 3. Perhaps a polymorphism within the *p63* gene itself is responsible for this effect.

Conclusions

In this study we present the results of mutation analysis in 227 patients with a p63-associated ED syndrome and their common phenotypic characteristics. An additional eight families are known with a causative *p63* mutation presenting with isolated SHFM. Our study documents 30 different mutations that can cause EEC syndrome, of which 5 are responsible for 86% of the patients. Most of the mutations that were found in the other syndromes differ from the EEC mutations and are specific for each of the respective disorders. In addition, we show that there is a specific phenotype for each of the five *p63* hotspot mutations. For example, there is a complete lack of cleft lip/palate in patients with a p.R227 mutation, who instead have a high incidence of urogenital problems. Another example is the mutation p.R304, which gives rise to orofacial clefts in 80% of the cases, whereas this occurs in only 40% for EEC syndrome as a whole. This clinical variability may cause difficulties in arriving at the correct clinical diagnosis, especially in single patients. Yet, there are also clear examples where one mutation can cause two different disorders, which is most obvious in p.R280 mutations, which are seen in EEC syndrome and in non-syndromic SHFM. This study did not identify any clear differences between the AEC and RHS syndromes. Phenotypes of these syndromes resemble each other very much, the main difference being the more frequent, but certainly not consistent occurrence of ankyloblepharon in AEC syndrome. Because these syndromes can be due to same mutations (32), we propose to group them into a single entity.

Acknowledgements

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Delineation of the ADULT syndrome phenotype due to arginine 298 mutations of the *p63* gene

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Abstract

The ADULT syndrome (Acro-dermato-ungual-lacrimal-tooth, OMIM 103285) is a rare ectodermal dysplasia associated with limb malformations and caused by heterozygous mutations in *p63*. ADULT syndrome has clinical overlap with other *p63* mutation syndromes, such as EEC (OMIM 604292), LMS (OMIM 603543), AEC (106260), RHS (129400) and SHFM4 (605289). ADULT syndrome characteristics are ectrodactyly, ectodermal dysplasia, mammary gland hypoplasia and normal lip and palate. The latter findings allow differentiation from EEC syndrome. LMS differs by milder ectodermal involvement. Here we report three new unrelated ADULT syndrome families, all with mutations of arginine p.298. On basis of 16 patients in five families with p.R298 mutation, we delineate the ADULT syndrome phenotype. In addition, we have documented a gain-of-function effect on the ΔNp63α isoform caused by this mutation. We discuss the possible relevance of oral squamous cell carcinoma in one patient, who carries this *p63* germline mutation.

Introduction

Various combinations of limb anomalies, ectodermal dysplasias and orofacial clefts characterize heterozygous mutations in the transcription factor gene *p63*. The prototype *p63* mutation syndrome is EEC Syndrome, which is characterized by Ectrodactyly, ectodermal dysplasia, and cleft lip/palate (OMIM 604292). Ectrodactyly, a severe median cleft in hands and feet is often associated with syndactyly in these patients, but syndactyly can also be the sole limb defect. Ectodermal dysplasia can be found in various combinations of sparse hair, dry skin, hypodontia, dysplastic nails and alterations in sebaceous glands, mammary glands and nipples. Orofacial clefting is the third hallmark of the EEC syndrome and usually manifests as cleft lip with cleft palate. Symptoms in the other five inherited syndromes with *p63* mutations are overlapping, but each of these syndromes has its own characteristic combination of phenotypic features. For instance, ankyloblepharon is almost limited to the Hay-Wells syndrome (Ankyloblepharon-ectodermal defects-cleft lip/palate, OMIM 106260). Mammary gland hypoplasia and nipple hypoplasia are frequent findings in Limb mammary syndrome (LMS, OMIM 603543) and in Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285) but are rare in other p63-related conditions (1). ADULT syndrome is further distinguished from other p63 syndromes by absence of orofacial clefting and by prominent ectodermal signs. Abnormal hair or skin abnormalities have not been reported among LMS patients. Facial clefting in LMS is of another type than that in EEC syndrome: cleft palate only, whereas no facial clefts have been found among reported ADULT families (2-6). In one family, extensive freckling has been reported to be a typical characteristic of ADULT syndrome, and this was also mildly present in two further ADULT syndrome patients (2,4,7).

The causative gene in all these disorders, *p63*, is crucial during embryonic development, mostly in the development of limbs and other ectodermal derived tissues. The pattern of mutations in six different *p63* related syndromes shows genotype-phenotype correlations. The most pronounced genotype-phenotype correlation is in the EEC and Hay Wells syndromes. All EEC missense mutations are clustered in the DNA binding domain and disrupt binding to DNA (8). In contrast, all missense mutations reported for Hay-Wells patients are in the Sterile alpha motif domain, and have been shown to disrupt interaction with other proteins (9). LMS and ADULT syndrome have their own unique mutated amino acid residues. In the original Limb mammary syndrome family a point mutation was found in the coding region of the second transactivation domain (p.G76W) (3).

Only two amino acid residues are known to be mutated among ADULT syndrome patients: asparagine p.6, which is in the putative second transactivation domain and arginine p.298, which locates in the DNA binding domain (4,5,7). Although p.R298 is in the DNA binding domain, it is functionally different from the EEC mutations, because its substitution by glutamine does not lead to a loss of DNA binding, but instead to a gain of transactivation activity of the ΔNp63γ isoform (6). Here we report three further ADULT syndrome families with p.R298 mutations, either a p.R298Q or p.R298G. We discuss the consistent phenotypic features associated with these gain-of-function mutations.

Materials and methods

Patients and families

In a small Finnish family three family members, one in each generation, present with an EEC-like disorder. Their limb malformations vary from severe four-limb ectrodactyly and syndactyly in the oldest person (Fig. 1A-B) to unilateral syndactyly in the child of the third generation.

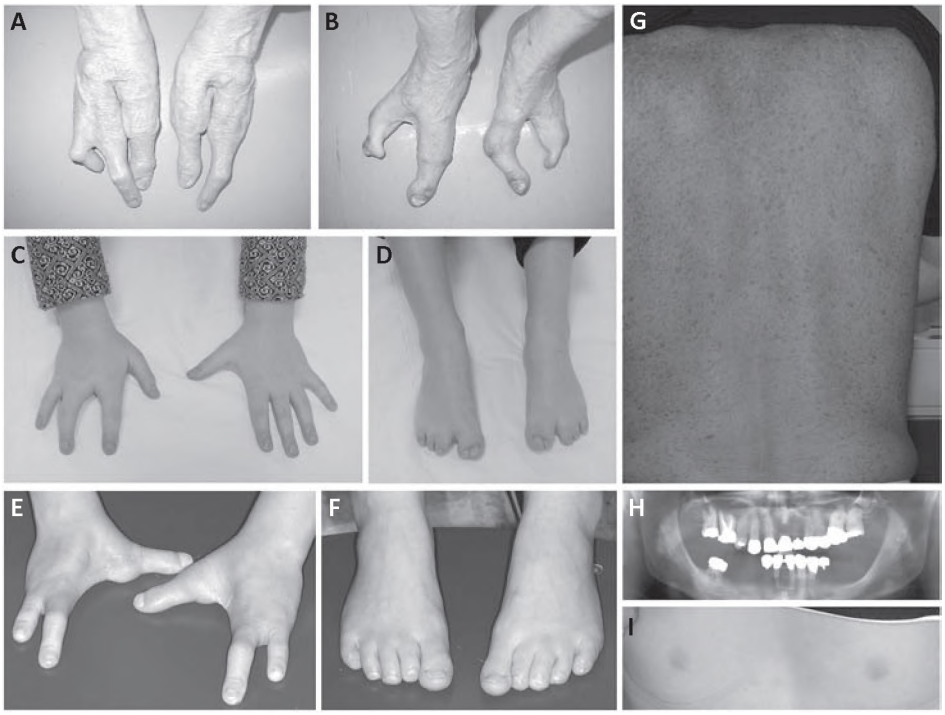

Figure 1. Limb and ectodermal phenotype in ADULT syndrome patients with p.R298 mutation. Limb malformations in p.R298 patients can vary much. **A-B**. Deep median cleft and syndactyly in the limbs of the Finnish ADULT syndrome patient (I/1) with p.R298G mutation. **C-D**. Mildly affected limbs in the Italian patient (II/1) with p.R298Q mutation. **E-F**. Severely affected hands and non-affected feet of Danish ADULT patient (II/1) with p.R289Q mutation. **G**. Excessive freckling in Danish patient (I/1). **H**. Hypodontia and loss of permanent teeth in Danish patient (I/1). **I**. Hypoplastic nipples in Danish patient (II/1). For a color figure see Appendix 2.

All patients have a similar pattern of malformations in ectodermal derived tissues as described in the original German ADULT syndrome family (2,4). Atrophic, dry skin is present, but freckling was not seen. Breast hypoplasia is found in adult female patients. The nine-year-old patient has only widely spaced nipples and congenital lacrimal duct obstruction. All affected individuals have very blond, thin and sparse hair. Nails are reported to be dysplastic, and one or several teeth were lacking. A malignant tumour in the mouth of one of the patients was diagnosed at age 60. Histological examination confirmed a squamous cell carcinoma (SCC). Otherwise the medical history is unremarkable for all patients.

In a small Italian family, mother and son have symptoms similar to those described above for the Finnish family, and previously reported for a German family (2,4). The son has absence of the middle finger in his right hand and cutaneous syndactyly in both feet together with right foot polydactyly (Fig. 1C-D). His mother has normal limbs. Both affected individuals have lightly pigmented, dry and very photosensitive skin. Dermatitis has occurred in both individuals. Both mother and son have dysplastic nails with pits. The mother has oligodontia (dysplastic and hypoplastic teeth), whereas the son has only small teeth. The mother also has breast hypoplasia and absence of the right nipple, and the son has hypoplastic nipples. The son has tear duct obstruction and his mother absence of tears and chronic conjunctivitis, probably due to obstructed tear ducts. The mother was recently diagnosed with autoimmune thyroiditis and paroxysmal supraventricular tachycardia. The maternal grandfather of the son died at the age of 36 from a clear-cell renal carcinoma. He had lightly pigmented hair and skin, and he was highly photosensitive, like his family members. Otherwise no EEC-like symptoms are reported for the grandfather.

In a Danish family, father and daughter have symptoms similar to those described above for the Finnish and the Italian family. Ectrodactyly and syndactyly is present in the hands and one foot of the father and ectrodactyly is present in the hands of the daughter (Fig. 1E-F). Both father and daughter have nail dystrophy in all nails. Their skin is dry and thin, and excessive freckling of the entire body surface is present (Fig. 1G). Father and daughter both have hypodontia and dysplastic teeth (Fig. 1H). The daughter has hypoplastic nipples (Fig. 1I). The hair is unaffected in this family. The father has blocked tear ducts, as has his daughter, who was operated on when she was a child. There is no orofacial clefting in these patients.

Mutation Analysis

From the Finnish family blood samples were obtained from three patients and the proband´s healthy sister after a written informed consent was obtained. Genomic DNA was extracted using a Nucleon Extraction & Purification Kit (Amersham Biosciences). The exons, which have been reported to contain most pathogenic mutations of , were amplified by PCR and the products were purified by GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and sequenced directly using an ABI PRISMTM 377 automated sequencer (Applied Biosystems). The observed sequence change was confirmed with a restriction fragment analysis using HaeIII enzyme (New England BioLabs). Fifty-four control persons were tested for the presence of the pathogenic mutation. Mutation analysis for the Italian family was performed as described by Duijf et al (6).

From the Danish family blood samples were obtained from two affected and two nonaffected members after a written informed consent was obtained. Genomic DNA was extracted from peripheral blood samples using a standard salting-out method.

All exons and exon-intron boundary regions were sequenced on both strands. Primers were removed by treatment with 1U shrimp alkaline phosphatase (USB, Cleveland, OH, USA) and 10U Exonuclease I (New England Biolabs) followed by sequencing using the BigDye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and analysed on an ABI 377 sequencer (Applied Biosystem).

Functional analysis of p.R298G mutation

We performed a transactivation assay to see whether the p.R298G mutation causes a gain-of-function effect for ΔNp63γ isoform similar to the p.R298Q-mutation (6). Mutagenesis and transactivation assays were performed as described previously (6). Site-directed mutagenesis for introduction of the p.R298G mutation was performed with oligonucleotide 5´-cgcaagtcctgggcggacggtgctttgag-3´ and its reverse complement as described previously (8).

Immunohistochemical staining

A four-micrometer-thick section was cut from the formalin-fixed paraffin-embedded oral mucosa SCC sample. Immunohistochemical staining was performed using a TechMate500 immunostaining machine and a peroxidase/diaminobenzine (DAB) multilink detection kit (DAKO, Denmark). The section was stained with 4A4 monoclonal antibody (1:180) (a gift from Dr. F. McKeon, Harvard, USA) and counterstained with Mayer's haematoxylin.

Results

Mutation analysis

Mutation analysis of the *p63* gene was carried out by direct sequencing of *p63* exons in the probands of the Finnish, the Italian and the Danish family. In the Italian and the Danish family a heterozygous transition from a guanine to an adenine was found at nucleotide position c.893. This mutation is located at the beginning of exon 8 and causes a change from CGA to CAA at codon p.298. This mutation is identical to one that was originally identified in the German ADULT syndrome family and predicts an amino acid change from arginine to glutamine (p.R298Q) (6,10). Later this mutation was also reported by Chan et al (7). In the Finnish family a novel *p63* mutation was found at the same codon p.298: a heterozygous point mutation changing a cytosine to a guanine at nucleotide position c.892, which creates a change from CGA to GGA at codon p.298 and a predicted amino acid change from arginine to glycine (p.R298G) in the DNA binding domain. Fifty-four control samples were tested for the mutation with a restriction fragment analysis with the restriction enzyme HaeIII, which cleaves the wildtype amplicon, but not the mutated DNA fragment. None of the control samples or healthy family members carried the p.R298G mutation. Both mutations fully segregated with the phenotype in the three families.

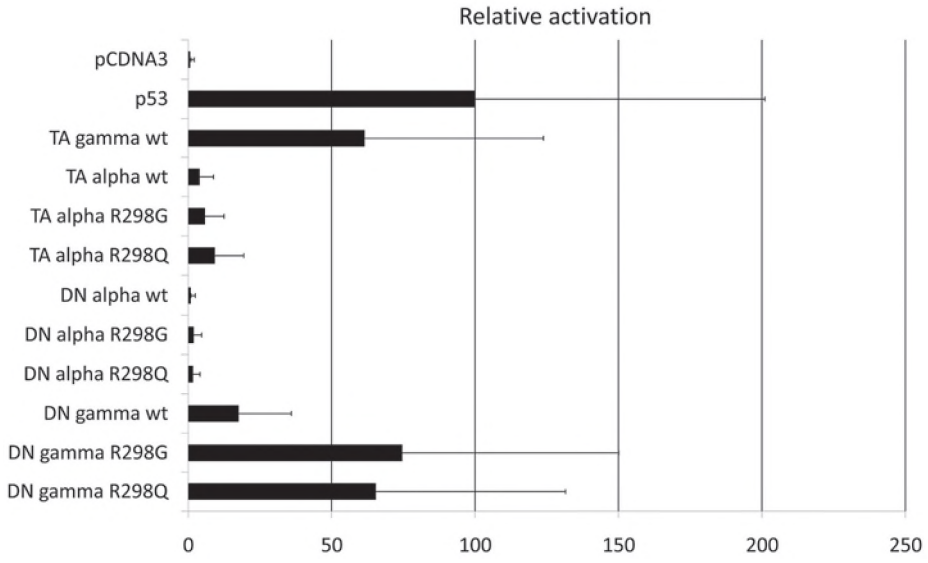

Figure 2. Transactivation of the wild-type p63, p.R298Q and p.R298G mutant isoforms. Transactivation assays are performed in extracts from SaOs-2 cells, which were transfected with wild-type, p.R298Q or p.R298G mutant constructs. The relative transactivation activity is given with respect to the p53 expression vector. TAp63γ wild-type is the only isoform, which is known to be able to transactivate p53 response elements. Here, both p.R298Q and p.R298G mutations cause a gain of activation on ΔNp63γ isoform, whereas the other mutated isoforms resemble the wild-type p63 behaviour. Transactivation activity data of TAp63γ p.R298Q/G mutation constructs is not shown.

Transactivation studies

According to the structural model of the DNA binding domain of p63, the arginine at position p.298 does not locate close to binding DNA (6). Indeed, transactivation studies have established that the p.R298Q mutation does not impair transactivation activity as do EEC causing mutations (6,8). Instead, the ΔNp63γ isoform carrying the p.R298Q mutation has highly increased transactivation activity as compared to its wildtype counterpart. To investigate whether this effect is specific for this amino acid substitution we decided to test the effect of the newly identified p.R298G mutation in transactivation assays. Functional analysis of the p.R298G mutation also yielded an exceptionally high transactivation activity, very similar to the activity, which was observed for the p.R298Q ADULT syndrome mutation (Fig. 2). It thus appears that the functional consequences of these mutations are comparable.

Immunohistochemical studies

Immunohistochemical staining using the 4A4 monoclonal antibody showed strong p63 accumulation in the undifferentiated squamous cell carcinoma of the p.R298G-patient (Fig. 3).

Table 1. ADULT syndrome characteristics caused by p.R298 mutations are compared to other allelic syndrome failures[a] and other reported ADULT syndrome features (p.N6H and p.R298Q) (2,4,5,7).

	Phenotypic features[a]					Finnish p.R298G			Italian p.R298Q		Danish p.R298Q		German p.R298Q								p.R298G	p.N6H
	EEC	AEC	RHS	LMS	ADULT	I/1 F	II/1 F	III/1 F	I/1 F	II/1 M	I/1 M	II/1 F	III/3 M	III/10 M	IV/2 F	IV/6 M	IV/8 F	IV/10 F	V/1 M	V/2 M	M	F
Limb																						
Hand malformation	++	+	+	++	++	+	+	+	-	+	+	+	+	+	+	-	-	-	+	-	+	+
Foot malformation	++	+	+	++	++	+	+	-	-	+	+	-	-	+	+	+	-/+	-	+	-	-	+
Polydactyly	-/+	-	-	-	-/+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-		
Skin																						
Dry and thin	+	++	++	-/+	+++	+	+	+	+	+	+	+									+	
Neurodermatitic signs					++	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	
Sweat glands	+	+	+	+	-/+	+	-	-	-	-	-	-									+	
Freckling	-	-	-	-	++	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	+/-	+
Photosensitivity	-	-	-	-	-/+	-	-	-	+	+	-	-										
Hair																						
Thin and sparse	++	+++	+++	-	++	+	+	+	-	-/+	-	-			+		+					
Alopecia (frontal)				-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-		
Nail																						
Dysplasia	++	++	++	++	+++	+	+	+[b]	+[bc]	+[bc]	+	+	+	+	WG	+	+	+	+	WG	+	+
Teeth																						
Hypodontia	++	++	+	+	++	+	+	+	+		+	+	-	-	+	-	+	-	-	-	+	+
Loss of permanent teeth					++	+	-	-	-		+	+	+	+	+	+	+	+				
Oligodontia					++	+	+	+	+	+[d]	+	+									+	
Breast and nipple																						
Hypoplasia	+	+	-/+	+++	++	+	+	WS	+	+	-	+		+	+	+		+	+		+	+
Eye																						
Lacrimal duct obstruction	++	++	++	++	++	+	-	+	-/+	+	+	+	(+)	+	-	+	+	+	-	-	+	+
Eyelid fusion	-	++	-/+	-	-	-	-	-	-	-	-	-										
Bilateral ptosis	-	-	-	-	-/+	+	-	-	-	-	-	-										
Conjunctivitis	+				+	+	-	-	+	+	-	-										
Orofacial clefting																						
Cleft palate	++	++	++	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cleft lip and palate	++	++	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Other																						
Albinoid appearance					-/+	-	-	-	(+)	+	-	-										
Otitis (ear infection)		+			+	-	-	-	+	+	-	-										
Mild face dysmorphism	+	+	+	-	+	-	-	-	-/+	+	-	-										+
Microretrognathia	+	+	+	-	-/+	-	-	-	-	+	-	-										

Note: +++ consistent, ++ frequently observed, + occasionally observed, -/+ rarly observed, - never observed. Blank fields: no data available.
[a]Based on a compilation of 227 patients by Rinne et al (1). F=female, M=male, WS=widely spaced, WG=watch glass nails, [b]=pits, [c]=brittle, [d]=small teeth.

Figure 3. Undifferentiated oral squamous cell carcinoma (SCC) of the Finnish ADULT syndrome (p.R298G) patient. The SCC oral mucosa (on the left) shows strong p63 immunoreactivity, whereas the p63 expression in the healthy mucosa is strongest in the basal layer and gradually decreased in the suprabasal layers (on the right). (Magnification is 200x.) For a color figure see Appendix 2.

Clinical evaluation of patients with a mutation at p.R298

Having established that the p.R298G mutation has similar functional effects as the p.R298Q mutation, we next draw a comparison of the clinical manifestations in the Finnish family with the p.R298G mutation and the patients with the p.R298Q mutation. The clinical observations in patients from the three families in this study are summarized in Table 1. These features are scored against the typical phenotypic symptoms of each of the six syndromes that are associated with *p63* mutations: EEC syndrome, AEC syndrome, Rapp-Hodgkin syndrome, LMS, ADULT syndrome, and SHFM. It appears that the phenotype of patients from the Finnish family is most similar to the ADULT syndrome phenotype. However, also some differences can be noted, such as the lack of neurodermatitic signs and the squamous cell carcinoma in one individual. The same is true for the Italian and the Danish families with the p.R298Q mutation. Patients from the Italian family have the typical hallmarks of ADULT syndrome, but differ because of the normal hair phenotype, conjunctivitis, photosensitivity and albinoid appearance. The Danish family differs from the original ADULT family because of the lack of hypohidrosis, but presents the excessive freckling.

Discussion

We have identified a *p63* mutation in three unrelated families affecting arginine p.298 in the DNA binding domain of p63. One mutation, p.R298G, was not detected before, but we have demonstrated that it is functionally equivalent to the p.R298Q mutation in two families diagnosed as ADULT syndrome, and to the second and third family reported here (2,6,7). Comparison of all 16 patients with a p.R298 mutation allows a better delineation of the ADULT syndrome phenotype.

Hand and/or foot malformations are observed in all ADULT patients except one (15/16). Ectodermal dysplasia is present in all individuals with p.R298 mutation and always affects the nails, teeth and skin. Nails are dysplastic, discoloured, brittle and/or have pits. Hypodontia, oligodontia and/or small teeth are a common combination, in addition to loss of permanent teeth in young adulthood. The skin is mostly dry and thin, sometimes with neurodermatitic signs. Also frequently observed are defects of the mammary glands, hair and lacrimal ducts. Mammary gland and/or nipple hypoplasia is present in 12 out of 16 patients, including males. Some ADULT patients show signs that may be family-specific traits. For example, photosensitivity and hypopigmentation are only seen in the Italian family. Also, the extensive freckling, which stands as one of the typical hallmarks of ADULT syndrome, is observed in the original ADULT syndrome family, in the Danish family, and a very mild version (freckling on cheeks) is reported in a sporadic ADULT syndrome case (2,7). Even in the original ADULT syndrome family the freckling is not a consistent feature. Therefore, it is possible that other genetic factors contribute to this part of the phenotype either as a major gene or as a modifier. Finally, oral SCC was seen in one of the patients, but its relation to the p63 p.R298Q mutation is obscure (see below).

In conclusion, ADULT syndrome is the most likely clinical diagnosis in a patient with ectrodactyly, mammary gland hypoplasia, neurodermatitic signs, prominent ectodermal dysplasia and normal lip and palate. This specific combination of clinical features is a-typical for EEC syndrome. However, the high degree of clinical variability in EEC syndrome may confuse a correct clinopathological diagnosis, especially for isolated patients. Presence of clefting or absence of mammary gland involvement is indicative of EEC syndrome. LMS is clinically very similar to ADULT syndrome, but differs by much milder ectodermal involvement. Absence of neurodermatitic signs such as freckling or hypopigmentation does not preclude a diagnosis of ADULT syndrome.

Having defined the common features associated with mutations at p.R298, it is of interest to compare these with the single ADULT syndrome patient with another mutation, p.N6H (Table 1) (5). The p.N6H patient has ADULT signs such as limb malformation, nipple hypoplasia and lacrimal duct obstruction, nail dysplasia and hypodontia. However, she does not have hair abnormalities, alopecia or skin involvement. This patient has freckling, but this was absent in two of the five families with p.R298 mutations. We believe that freckling may not be a discriminating factor in ADULT syndrome characteristics. Altogether p.N6H mutation causes a syndrome, which equally resembles ADULT syndrome and Limb mammary syndrome. Thus, this patient cannot be confidently classified as either ADULT or LMS. The location of the p.N6H mutation, in the beginning of ΔNp63, could indicate LMS syndrome, since the p.G76W mutation is only 15 amino acids downstream in ΔNp63. (The numbering of these mutations is based on different p63 isoforms.)

We have shown the functional consequences of the p.R298 mutation in vitro. This mutation induces transactivation activity for an ΔNp63γ isoform, which usually does not have this capacity. Although the mechanism of the gain-of-function effect is still unclear, this observation has led to the identification of a putative second transactivation domain uniquely present in the ΔN-isoforms (6). Here, we have shown that the gain-of-function effect is not specific to p.R298Q. The p.R298G substitution has the same functional effect. This result indicates that the mechanism of these mutations depends on the removal of arginine p.298, and consequently the disruption of p63 properties that are mediated through this amino acid, and not so much by novel properties that are brought about by the substituted amino acid.

Since *p63* is a family member of the well-known tumour suppressor gene *p53*, it was suspected to have a role in cancer development from its discovery. There are several reports of strong p63 accumulation in basal cell carcinomas and squamous cell carcinomas (11-15). Also, the undifferentiated squamous cell carcinoma (SCC) of the mouth of the ADULT patient (p.R298G) described in this article showed strong p63 accumulation (Fig. 3). A clear-cell-renal carcinoma was reported for the maternal grandfather of the Italian family, but we were unable to establish whether he was a carrier of the p.R298Q mutation. So far only one EEC patient with *p63* mutation (p.D312G) has been described to have a cancer, a malignant B-Cell Lymphoma (16). These are the only reported descriptions of human p63 patients developing cancer, suggesting that individuals with a germline *p63* mutation have little or no increased risk to develop cancer. A recent study with heterozygous *p53, p63* and *p73* knockout mice studies reported that the life span for each genotype was reduced and incidence of the tumours was increased (17). Loss of heterozygosity of each of the p53 family members was observed in these tumours. Interestingly, squamous cell carcinoma was observed in ten percent of heterozygous *p63* mutant mice. Complete loss of heterozygosity in this cancer type further suggested a tumour-suppressor role for p63. This observation is in sharp contrast to previous suggestions that p63 acts as an oncogene and accumulates in tumours, notably in SCCs as in the present patient with an p.R298Q mutation (11-15). An oncogenic activity for p63 would also be in line with the gain-of-function activity that is brought about by the p.R298 amino acid substitutions. Nevertheless, since SCCs are common (7-11% lifetime risk (18)) we cannot conclude that there is a causal relationship between the SCC and the *p63* mutation in the present ADULT syndrome patient.

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Spectrum of *p63* mutations in a selected patient cohort affected with Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC)

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Abstract

Heterozygous mutations in the *p63* gene underlie a group of at least seven allelic syndromes, including Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC) and Rapp Hodgkin syndrome (RHS), which involves varying degrees of ectodermal dysplasia, orofacial clefting and limb malformations. Mutations in the AEC and Rapp Hodgkin syndromes cluster in the 3' end of the *p63* gene. Previously reported mutations are mainly missense and frameshift mutations in exons 13 and 14, affecting the p63α-specific SAM (Sterile alpha motif) and TI (Transactivation inhibitory) domains. A patient cohort affected by AEC syndrome was evaluated during International Research Symposium supported by the National Foundation for Ectodermal Dysplasias. Nineteen patients underwent full clinical evaluations and 18 had findings consistent with a diagnosis of AEC syndrome. These 19 patients, along with 5 additional relatives had genomic DNA analysis. Twenty-one of the 24 participants from 12 families were found to have mutations in the *p63* gene. Eleven different mutations were identified; 10 were novel mutations. Eight were missense mutations within the coding region of the SAM domain. Three other mutations were located in exon 14 sequences, which encode the TI domain. The effects of the mutations in the SAM and TI domains are poorly understood and functional studies are required to understand the pathological mechanisms. However, AEC and RHS mutations in the 5' and 3' ends of the *p63* gene point towards a critical role of the Δp63α isoform for the AEC/RHS phenotype.

Introduction

Heterozygous mutations in the *p63* gene (TAp63 mRNA, Genbank AF075430) underlie a group of at least seven human developmental disorders, characterized by different combinations of ectodermal dysplasia, orofacial clefting and limb malformations (1-7). Manifestations in these syndromes are overlapping, but different enough to be considered discrete syndromes. Some of these syndromes present a strong genotype-phenotype correlation, whereas correlation in the other syndromes is more ambiguous. Hay-Wells syndrome, also known as Ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260), and Rapp Hodgkin syndrome (RHS, OMIM 129400) are highly similar disorders and possibly variable manifestations of the same clinical entity (8,9). Typical characteristics for AEC and RHS are ankyloblepharon filiforme adnatum (eye lid fusion), severe skin erosion at birth and abnormal hair (pili torti or pili canaliculi) and the presence of cleft palate with or without cleft lip. Severe limb malformations such as ectrodactyly are less commonly observed in these conditions.

Mutations in AEC and RHS syndromes cluster in the 3' end of the *p63* gene. These mutations are mainly missense and frameshift mutations in exons 13 and 14, affecting the p63α-specific SAM (Sterile alpha motive) and TI (Transactivation inhibitory) domains. Although, AEC and RHS syndromes have always been linked to the α-tail of the *p63* gene, we have recently discovered three novel mutations in the 5' end of the *p63* gene (10). Two of these novel mutations only affect the ΔNp63-isoforms (not the longer TAp63-isoforms), indicating that the specific disruption of the ΔNp63α-isoform is the key to the AEC and RHS syndrome phenotypes.

Table 1. ***p63*** **mutations identified in 24 patients of NFED cohort.**

Sample	Mutation (DNA)	Mutation (protein)	Exon	Domain
1	c.1630G>T	p.D544Y#*	14	SAM
2	c.1793G>T	p.R598L*	14	TI
3	c.1682G>A	p.G561D*	14	SAM
4	c.1682G>T	p.G561V*	14	SAM
5	c.1793G>T	p.R598L*	14	TI
6	c.1682G>A	p.G561D*	14	SAM
7	c.1793G>T	p.R598L*	14	TI
8	Unaffected family member			
9	c.1802A>T	p.D601V*	14	TI
10	c.1682G>A	p.G561D*	14	SAM
11	Unaffected participant			
12	c.1793G>T	p.R598L*	14	TI
13	c.1578C>A	p.F526L*	13	SAM
14	c.1793G>T	p.R598L*	14	TI
15	c.1631A>T	p.D544V*	14	SAM
16	c.1652C>T	p.P551L*	14	SAM
17	c.1578C>A	p.F526L*	13	SAM
18	c.1634T>C	p.L545P*	14	SAM
19	c.1682G>T	p.G561V*	14	SAM
21	c.1610T>C	p.I537T	13	SAM
22	c.1846delC	p.R616fs*	14	TI
23	c.1846delC	p.R616fs*	14	TI
24	Unaffected family member			
25	c.1846delC	p.R616fs*	14	TI

Domain abbreviations: SAM (Sterile alpha motif) and TI (Transactivation inhibitory domain).
This mutation likely affects the splicing of exon 14.
*Novel *p63* mutations, which are not published before.

Materials and methods

The National Foundation for Ectodermal Dysplasias convened the International Research Symposium for Ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome at Texas Children's Hospital in Houston, TX, with financial support through the NIH. Nineteen patients with a suspected diagnosis of AEC syndrome underwent full multidisciplinary evaluations via a Baylor College of Medicine IRB-approved protocol. Eighteen of these patients were found to have clinical characteristics consistent with a diagnosis of AEC syndrome; one did not have a phenotype that was consistent with this diagnosis. An additional five relatives also participated, but their participation was limited to laboratory investigation only. In total, 24 participants from 12 different families had genomic DNA analysis to assess for mutations in the *p63* gene. Since all known AEC/RHS causing mutations have been found in exons 3', 4, 13 and 14 in the *p63* gene, these exons were investigated by direct sequencing. The mutation analysis was described elsewhere (3).

Results and discussion

Mutations were identified in 21 samples (Table 1). Three individuals did not have an identified mutation in the *p63* gene. Two of these are unaffected relatives, and one is a patient with a phenotype slightly different than AEC/RHS. This individual may have mutation elsewhere in the *p63* gene or in another yet unknown ectodermal dysplasia gene.

The number of solved patients from this NFED cohort is remarkably high, as our previous studies showed causative *p63* mutations in only ~75% of patients with an AEC/RHS like phenotype. Altogether, we found 11 different mutations, of which only one mutation (in patient 21) p.I537T has been described previously in AEC syndrome families (5,11,12).

Eight of the mutations reported here (p.F526L, p.I537T, p.D544Y, p.D544V, p.L545P, p.P551L, p.G561D and p.G561V) are missense mutations within the coding region of the SAM domain (Fig. 1). Such mutations are characteristic for AEC/RHS, since 57% (16 out of 28) of the known AEC/RHS mutations create amino acid substitutions in the SAM domain (5,8,9,11,13-17). However, two of these missense mutations (p.D544Y, p.D544V) are flanking the intron 13-exon 14 boundary and may cause a splice site defect of exon 14. In patient 1 the first nucleotide of codon p.544, guanine at nucleotide position c.1630 is changed to thymine; and in patient 15, the second nucleotide of this codon, adenine at nucleotide position c.1631 is changed to thymine. To predict the influence of these mutations, we tested these sequences in two splicing prediction programs (NetGene2 and Berkeley Drosphila Genome Project). The prediction programs suggest that the c.1630G>T mutation (p.D544Y) is most likely affecting the acceptor splice site of exon 14, whereas the c.1631A>T mutation (p.D544V) does not. Unfortunately, the consequences on the *p63* cDNA level cannot be determined since the patient cDNA is not available.

Three other new mutations are located in exon 14 sequences, which encode the TI domain of the p63 protein (Fig 1). In total, about 18% (5 out of 28) of previously identified AEC/RHS mutations are deletions in the TI domain causing a frameshift and an extended protein product (3,9,11,18-22). The newly identified p.R616fs mutation has a similar predicted effect.

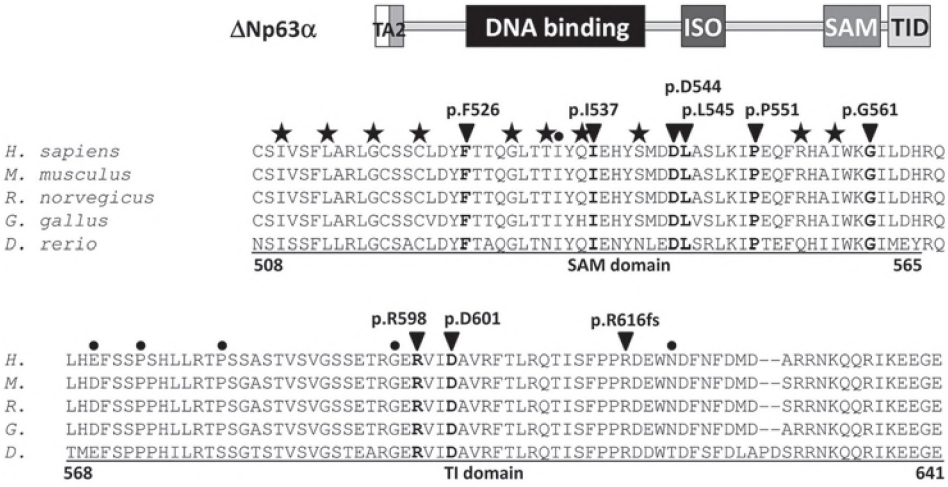

Figure 1. Multiple sequence alignment of p63α-tail represents the highly conserved SAM and TI domains. The nine amino acids, which were found to be mutated in this study, are indicated by black arrow and amino acid number. These amino acids are much conserved, which suggest their role as pathogenic mutations. Stars illustrate known missense mutations and dots known deletions or insertions. In total, 18 amino acids are known to be mutated in SAM and TI domains, and seven deletions or insertions are reported.

More surprising is the detection of two missense mutations, p.R598L and a p.D601V, which are located in the middle of the TI domain. These are the first missense mutations in the TI domain. Including this study, a total of 38 different mutations in AEC/RHS has been reported. Mutations are clustered in the ΔN-specific amino-terminus and α-specific carboxy-terminus of the p63 protein, which points towards a critical role of ΔNp63α isoform for the AEC/RHS phenotype and pathogenesis. Moreover the expression pattern in epithelial tissues of ΔNp63α is in agreement with the phenotypic malformations in AEC/RHS patients.

Functional consequences of AEC/RHS mutations have been reported in a few studies. One very likely effect is distorted binding of mutant SAM domain to its interacting proteins and its diverse consequences. It has been reported that SAM domain mutations abolish binding to Apobec1-binding protein 1 (ABBP1). ABBP1 belongs to the RNA processing machinery and controls splicing of Fibroblast growth factor receptor 2 (FGFR2), which is likely to be altered because of mutations in SAM domain (23). Another possible effect is linked to transactivation. Since ΔNp63α protein can act as an activator or a repressor, it is likely that AEC/RHS mutations can alter the repressor or activator function of the p63α protein (24-27). Our recent study shows that AEC/RHS causing mutations in the 5' end of the *p63* gene lose the activator function of ΔNp63α, and have even dominant negative activity against the wild type p63 protein (10). Mutations in the TI domain might also influence the repressor function. While these effects have been shown for some of the known AEC/RHS mutants, other mutations may cause other still unknown consequences affecting the same pathway and causing the same disease.

Acknowledgements

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Web resources

Online Mendelian Inheritance in Men (OMIM), http://www.ncbi.nlm.nih.gov/Omim/. GenBank, http://ncbi.nlm.nih.gov/GenBank (Accession number is AF075430 in text, Table 1 and Figure 1.) Splice site prediction sites: NetGene (http://www.cbs.dtu.dk/services/NetGene2/) and BDGP (http://www.fruitfly.org/seq_tools/splice.html).

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A novel translation re-initiation mechanism for the *p63* gene revealed by amino-terminal truncating mutations in Rapp-Hodgkin/ Hay-Wells like syndromes

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Abstract

Missense mutations in the 3' end of the *p63* gene are associated with either RHS or AEC syndrome. These mutations give rise to mutant p63α protein isoforms with dominant effects towards their wild type counterparts. Here we report four RHS/AEC-like patients with mutations (p.Q9fs, p.Q11X, p.Q16X), that introduce premature termination codons in the N-terminal part of the p63 protein. These mutations appear to be incompatible with the current paradigms of dominant-negative/gain-of-function outcomes for other *p63* mutations. Moreover it is difficult to envisage how the remaining small N-terminal polypeptide contributes to a dominant disease mechanism. Primary keratinocytes from a patient containing the p.Q11X mutation revealed a normal and aberrant p63-related protein that was just slightly smaller than the wild type p63. We show that the smaller p63 protein is produced by translation re-initiation at the next downstream methionine, causing truncation of a non-canonical transactivation domain in the ΔN-specific isoforms. Interestingly, this new ΔΔNp63 isoform is also present in the wild type keratinocytes albeit in small amounts compared to the p.Q11X patient. These data establish that the p.Q11X-mutation does not represent a null-allele leading to haploinsufficiency, but instead gives rise to a truncated ΔNp63 protein with dominant effects. Given the nature of other RHS/AEC-like syndrome mutations, we conclude that these mutations affect only the ΔNp63α isoform and that this disruption is fundamental to explaining the clinical characteristics of these particular ectodermal dysplasia syndromes.

Introduction

The p53 protein and its evolutionary predecessors p63 and p73 constitute a family of key transcriptional regulators in cell growth, differentiation and apoptosis. While *p53* is a major player in tumorigenesis, *p63* and *p73* appear to have pivotal roles in embryonic development. *p73*-deficient mice have neurological and inflammatory problems, whereas *p63*-knockout mice have major defects in epithelial, limb and craniofacial development (1-3). These observations suggest that *p63* has a crucial role in tissue morphogenesis and maintenance of epithelial stem cell compartments. Furthermore, *p63* has been linked to several important signaling pathways, such as Epidermal growth factor (EGF), Fibroblast growth factor (FGF), Bone morphogenetic protein (BMP) and Notch, WNT and Hedgehog (4-9).

The *p63* gene consists of 16 exons located on chromosome 3q28. At least six different protein isoforms can be produced, due to two different promoter sites and three different splicing routes. The amino-terminal ends are called TA and ΔN, and at the carboxy-terminal end, α, β and γ termini can be synthesized (Fig. 1). Several functional domains have been identified. The central DNA-binding domain and isomerization domain are present in all p63 isoforms. The canonical transcription activation (TA) domain is located at the amino-terminal end of the TAp63 isoforms. The ΔN-isoforms also contain an amino-terminal transactivation domain, denoted TA2 (10,11). The carboxy-terminal end has two additional domains: the Sterile alpha motif (SAM) domain and a Transactivation inhibitory (TI) domain, which are both only present in the largest carboxy-terminal variant, p63α (12).

Heterozygous mutations in the human *p63* gene cause developmental disorders, characterized by various combinations of ectodermal dysplasia, limb malformations and orofacial clefting (13,14). To date, seven different disorders have been linked to mutations in the *p63* gene (15).

These conditions may have overlapping phenotypic features, but some genotype-phenotype correlations have emerged (16). EEC syndrome (Ectrodactyly, ectodermal dysplasia and cleft lip/palate, OMIM 604292) is the most common p63-linked ectodermal dysplasia (ED). It is characterized by three major clinical symptoms: cleft lip and/or palate, ectodermal dysplasia (abnormal teeth, skin, hair, nails and sweat glands) and limb malformations in the form of split hand/foot (ectrodactyly) and/or fusion of fingers/toes (syndactyly). About 10% of p63-linked patients have Rapp-Hodgkin syndrome (OMIM 129400) or AEC/Hay-Wells syndrome (OMIM 106260). These two latter syndromes fulfill the criteria of ectodermal dysplasia and orofacial clefting, but do not have the severe limb malformations seen in EEC syndrome. Some typical characteristics linked to RHS/AEC-like syndromes are eyelid fusion (ankyloblepharon filiforme adnatum), severe skin erosion at birth and abnormal hair with pili torti or pili canaliculi. Indeed, RHS and AEC syndromes are very similar and have been suggested to be variable manifestations of the same clinical entity (15,17). EEC and RHS/AEC syndromes are good examples of a strong genotype-phenotype association. Mutations in the EEC syndrome are clustered in the DNA binding domain, and most likely alter the DNA-binding properties of the protein. By contrast, mutations in RHS/AEC syndromes are clustered in SAM and TI domains in the carboxy-terminus of p63α (15-29). The SAM domain is involved in protein-protein interactions, whereas the TI-domain can bind intra-molecularly to the TA-domain, thereby inhibiting transcription activation (12,30). All p63-linked disorders are inherited in an autosomal dominant manner and mutations are thought to have either dominant-negative or gain-of-function effects (31).

In this article we describe four patients from three families with phenotypes consistent with RHS/AEC syndromes that result from atypical nonsense mutations (p.Q11X, p.Q16X) or a deletion mutation (p.Q9fs), all of which lead to a premature termination codon (PTC) in the early amino-terminal end of the ΔNp63-isoforms. These mutations are predicted to give rise to a null allele, which contradicts the accepted disease mechanism of other mutations.

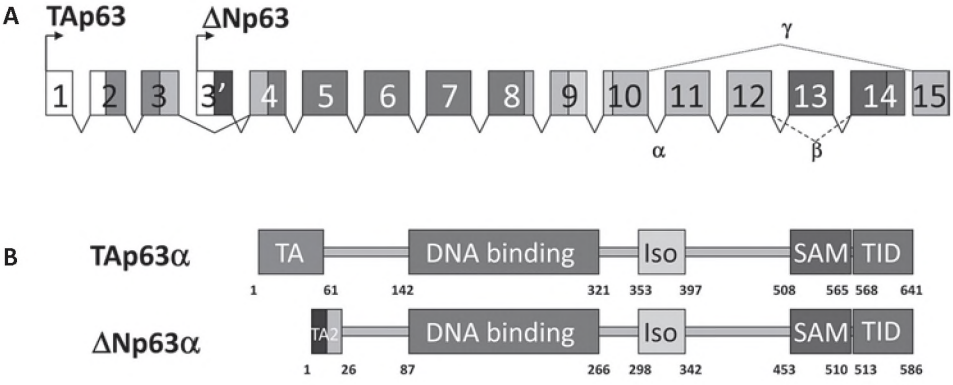

Figure 1. Schematic overview of the *p63* gene and two of its protein products. A. *P63* has two different promoter sites resulting in two different N-terminal ends called TA and ΔNp63. ΔNp63 lacks the exons 1-3, which are present in TA, however it contains an alternative exon 3'. *P63* has three C-terminal splicing routes: α, β and γ, thus at least six different gene products can be transcribed. **B.** The domain struc+ture of TA and ΔNp63 is similar, only N-terminal transactivation domain is different, called TA and TA2 respectively. Domain abbreviations: ISO (Isomerization domain), SAM (Sterile alpha motif domain) and TID (Transactivation inhibitory domain). The numbering under the domains illustrates the amino acid positions.

Surprisingly, in keratinocytes from the patient with the p.Q11X mutation we were able to detect normal p63 RNA levels of both alleles, but also an additional smaller protein product. Using molecular studies we could demonstrate that the smaller p63 protein was produced by translational re-initiation at the next methionine after the PTC. This causes a deletion of 25 amino acids in the ΔNp63-isoforms, abrogating the TA2 domain, and thus suggesting a crucial role for this part of p63 in the pathogenesis of RHS/AEC syndromes.

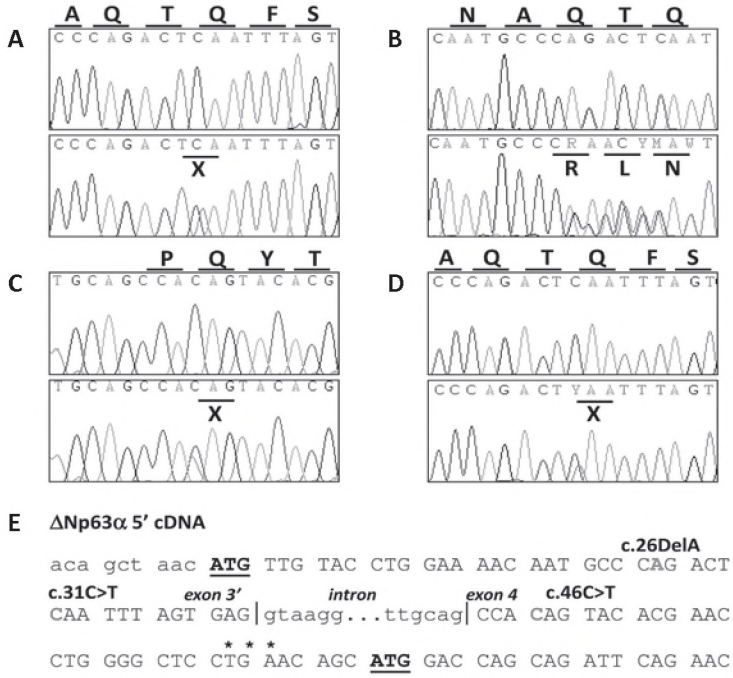

Figure 2. Pathogenic mutations in three RHS/AEC syndrome families. Direct sequencing of genomic DNA from four AEC/RHS patients revealed N-terminal mutations in the *p63* gene. **A.** In family 1 a heterozygous nucleotide change c.31C>T was found in affected mother and daughter in exon 3'. The upper chromatogram illustrates a control sequence and the lower is from the affected mother. **B.** In family 3, a heterozygous deletion c.26delA was detected in the index patient in exon 3'. The upper sequence is a control and the lower is from the patient. **C.** In family 2 a heterozygous nucleotide change c.46C>T (ΔNp63-isoform) was detected in exon 4 in the index patient. The upper chromatogram illustrates a control DNA sequence and the lower is from the patient. **D.** Chromatogram of the sequenced cDNA of keratinocytes from the mother of family 1 reveals the same heterozygous nucleotide change detected in the genomic DNA (Fig. 2A). Mutated RNA is present in the cells and is not degraded by the nonsense mediated RNA decay as expected. **E.** The translated sequence of ΔNp63α (AF075431) (capitals) contains two AUG sites (bold, underlined) in the two first exons (exon 3' and 4, | indicates the exon boundary). The mutated nucleotides: c.26A, c.31C and c.46C (indicated red and bold) are located between these two initiation sites. c.26delA mutation leads to a frameshift, which causes a PTC (indicated by ***) only 5 nucleotides upstream of the second AUG site. c.31C>T changes the codon CAA into a termination codon TAA (this PTC is 44 nucleotides upstream from the second AUG). c.46C>T changes codon CAG into a termination codon TAG causing a PTC 29 nucleotides upstream of the second AUG. The second initiation codon is flanked by a strong Kozak sequence, where the most important nucleotides (purine at position -3 and a guanine at position +4) are conserved suggesting its use in translation re-initiation. For a color figure see Appendix 2.

Identification of atypical mutations predicting N-terminal premature stop codons

We identified four patients in three families with a clinical presentation reminiscent of AEC/RHS like ectodermal dysplasia syndrome (for details see Material and methods). Direct sequencing of all 16 exons of the *p63* gene revealed three different heterozygous nucleotide changes in affected individuals. In family 1, both mother and daughter have a nucleotide change c.31C>T (Fig. 2A) (Accession number AF075431) in the first coding exon, the alternative exon 3', that is present only in the ΔNp63 isoform. The c.31C>T mutation changes glutamine to a stop codon at amino acid position 11, creating a premature termination codon (PTC) in all ΔNp63 isoforms.

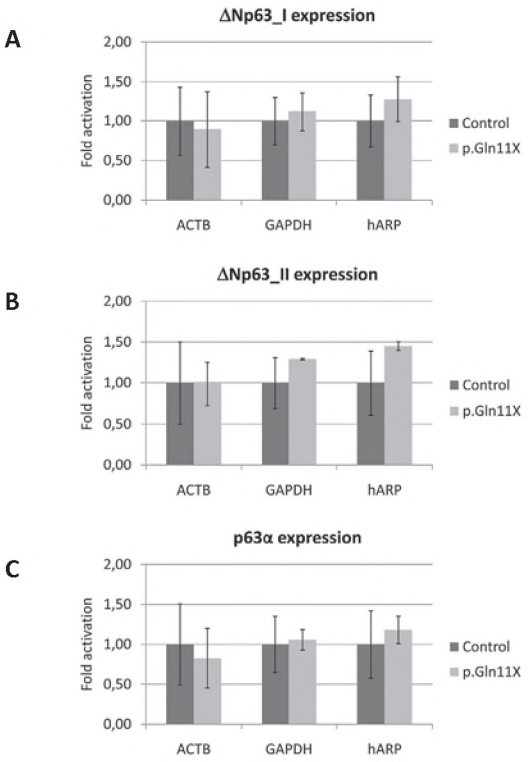

Figure 3. ΔNp63α levels in cultured keratinocytes from a Rapp-Hodgkin syndrome patient. ΔNp63 and p63α expression levels are investigated in undifferentiated keratinocytes by quantitative PCR. The control (black bar) is a pool of five control samples and the RHS patient sample (grey bar) is from the mother of family 1 containing the p.Q11X mutation. Housekeeping genes *ACTB*, *GAPDH* and *hARP* were used in normalization. **A-B.** qPCR with two ΔNp63 specific primer sets show that ΔNp63 expression in RHS sample is very similar to control, indicating that RNA containing the mutation is not degraded by NMD as expected. **C.** qPCR with p63α specific primer set gives similar results than ΔNp63 specific sets. Furthermore, similar Ct-values (not shown) indicate ΔNp63α to be the only isoform expressed in undifferentiated cultured keratinocytes.

In family 3, a deletion of one nucleotide (c.26delA) was identified (Fig. 2B), also located in the alternative exon 3' of ΔNp63 isoforms. This causes a frameshift and a PTC 41 nucleotides downstream from the deletion (Fig. 2E). In family 2, a nucleotide change c.46C>T was detected in exon 4 (Fig. 2C). This mutation causes a change of glutamine to a stop codon at amino acid position 16 in the ΔNp63 isoforms and at position 71 in TAp63 isoforms. The genomic change c.46C>T (or c.211C>T in TAp63 accession number AF075430) indicates a PTC in both DN and TAp63 isoforms. These three mutations were not identified in DNA from any of the unaffected family members nor in 300 control DNAs, indicating that they are all pathogenic, resulting in RHS/AEC-like syndromes.

Harmful and deleterious transcripts with a PTC are usually degraded through nonsense-mediated decay (NMD). In general, all transcripts containing a PTC upstream of the last exon junction complex are recognized and degraded (32). The c.31C>T mutation is present in the first exon of ΔNp63 followed by 11 exon junctions and c.26delA causes a PTC in the second exon (exon 4), followed by 10 exon junctions. c.46C>T (c.211C>T) causes a PTC in the second exon of ΔNp63 and in the fourth exon of TAp63, in both cases following 10 exon junctions. According to this paradigm, the transcripts with PTCs caused by these mutations should all be degraded by NMD and thereby cause *p63* haploinsufficiency.

To investigate this phenomenon in more detail we obtained a skin biopsy from the affected mother of family 1 and established a keratinocyte culture. p63 RNA expression levels in cultured keratinocytes were determined by quantitative PCR (qPCR) using two different primer sets specific for the 5' end encoding ΔN-isoforms and one primer set for the 3' end of the α-isoforms. All these three primer sets gave similar Ct-values suggesting that the major isoform in normal control keratinocytes is ΔNp63α (data not shown). Surprisingly, p63 RNA expression levels in cultured keratinocytes from the patient with the p.Q11X mutation were similar to those of controls (Fig. 3), which refutes the *p63* haploinsufficiency model. We then sequenced the amplified RT-PCR products, which revealed that the nucleotide change c.31C>T was also present in the keratinocyte RNA pool. The presence of both alleles (Fig. 2D) argues against NMD.

N-terminal truncation of ΔNp63alpha due to translational re-initiation

Having established that the nonsense mutations do not have an effect on the mutant transcript levels, we next determined the consequences of the N-terminal mutations at the protein level. Western blot analysis performed on protein extracts from the patient's cultured keratinocytes with an antibody specific to the α-tail of p63, revealed an additional ΔNp63 protein of reduced molecular weight (3kDa). A band of similar size was also present at very low levels in keratinocytes from control individuals, shown with the p63α-specific antibody in the Western blot in figure 4A. To resolve the identity of the smaller protein product, we investigated the nucleotide sequence downstream of the mutation. A further ATG codon was identified 44 nucleotides downstream of the c.31C>T mutation (Fig. 2E). This is the first ATG codon following the canonical ΔN start codon and is located 75 nucleotides downstream in the same reading frame. The Kozak sequence flanking this second AUG is in accordance with a strong translation initiation sequence, stronger even than the first AUG codon (Fig. 2E) (33,34). In addition, a translation start prediction program estimated this AUG to be an initiation site at score 0.631, a value within normal range of bona fide translation (Netstart 1.0 Prediction Server). We hypothesized that the next methionine

downstream of the p.Q11X mutation will be used to reinitiate the translation. A similar mechanism to escape NMD has been demonstrated previously for nonsense or frameshift mutations in *BRCA1*, *ATP7A* and *NEMO* genes (35-39).

To provide further support for the translation re-initiation hypothesis, we performed transfection studies in *p63* negative Saos-2 cells. We performed transient transfections with a full length ΔNp63α cDNA under a constitutive CMV promoter, pcDNA3_CMV_ΔNp63α. After transfections, the expression of each construct was confirmed by immunofluorescent labeling (data not shown). Protein lysate of each transfection was analyzed by Western blotting. Firstly, we mutated the second methionine at position p26 to isoleucine to investigate whether this methionine is involved in translation initiation of the smaller ΔNp63α fragment. Wild type ΔNp63α is present in two protein bands, however, when methionine at position p.26 is mutated, only the full length ΔNp63α protein was present (Fig. 4B) and the smaller protein variant was undetectable, even upon high over-exposure of the blot. Secondly, all three pathogenic mutations (p.Q11X, p.Q16X and p.Q9fs) and double mutants (p.Q11X_M26I, p.Q16X_M26I, p.Q9fs_M26I) were introduced into the ΔNp63α constructs. The transfection studies demonstrated a molecular size reduction (approximately 3kDa) after introduction of the p.Q11X, p.Q16X or p.Q9fs mutation into cells (Fig. 4C), which is accordance with the additional protein observed in the patient's keratinocytes (Fig. 4A). Finally, when introducing the double mutant p.M26I in combination with each pathogenic mutation, none of the two protein variants were detected (Fig. 4C). This strongly suggests that methionine p.26 is used to initiate the translation of ΔΔNp63α in the presence of mutations that cause upstream premature stop codons. The shorter protein variant ΔΔNp63α was also detected in cells transfected with wild-type ΔNp63α, but at lower levels than the full length protein. This phenomenon is similar to keratinocytes from control individuals, although the shorter variant is present in much lower levels compared to transfected cells.

Transcriptionally inactive ΔΔNp63alpha

The protein analysis showed that the p.Q11X mutation causes an amino-terminal deletion of 25 amino acids. To study the transactivational activity of the ΔNp63 amino-terminus, we tested a series of truncation mutations in the ΔNp63γ isoform (Δ14, Δ26, Δ43, Δ61, Δ79) for transactivation activity (Fig. 5A). Full length ΔNp63γ was able to activate an optimized p53 promoter nearly 6-fold more than the empty vector. In contrast, truncation of the first 14 amino acids completely abolished the activation (Fig. 5B). The same inactivating effect was detected for all other truncation mutations. Thus, 14 unique amino acids at the ΔN-terminus of p63 constitute a functional domain, which has transactivational activity. This is in accordance with previous studies on other promoters (10,11,40).

Because ΔNp63α is the predominant isoform in keratinocytes, we set out to test the ability of the p.Q9fs, p.Q11X and p.Q16X mutants to regulate downstream target genes. *Keratin-14* (*KRT14*) was recently reported to be a natural target gene of p63, which is upregulated by ΔNp63α (40-42). A transactivation assay of p63-negative Saos-2 cells transfected with either ΔNp63α wild type, mutant or a combination of these constructs, together with a KRT14-luciferase-reporter construct, revealed that wild type ΔNp63α was able to activate the KRT14 promoter 2.5 times more than the empty vector. This is in contrast to all ΔNp63α mutant constructs (p.Q9fs, p.Q11X and p.Q16X), which were inactive and behaved similar to empty vectors (Fig. 5C). Co-transfections of each single mutation in combination to ΔNp63α wild type vector showed no increased

KRT14-promoter activation (Fig. 5C), indicating a dominant effect of these mutations against the wild type. Next we investigated whether the mechanism of action of the mutant ΔNp63α protein might be dose dependent. To that end, we co-transfected Saos-2 cells with the wild type and mutant constructs in a 5:1, 1:1 and 1:5 ratio (Fig. 5D). The 5:1 ratio of wild type and p.Q11X mutant was able to activate the KRT14-promoter 2.3 fold, which is similar to the wild type ΔNp63α activation. In contrast, when we increased the amount of mutant p.Q11X construct the activation was reduced till 1.2 and 0.8 in 1:1 and 1:5 ratios respectively, indicating a dose-dependent inhibitory activity. Finally, we tested the dominant effect of mutant ΔNp63α_p.Q11X towards TAp63γ isoform (Fig 5E). ΔNp63α wild type can inhibit the TAp63γ activation from nearly 35-fold till 5-fold when compared to empty vector. The mutated ΔNp63α_p.Q11X has a similar inhibitory activity as the wild type ΔNp63α, indicating that the mutated protein can bind to DNA. These results show that mutant ΔNp63α isoforms have dominant effects towards various wild type p63 isoforms and that they are dose-dependent.

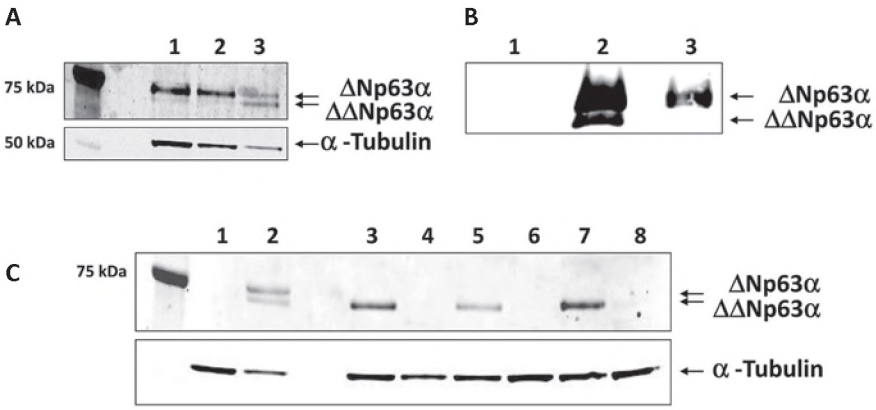

Figure 4. An additional ΔNp63α protein product in Rapp-Hodgkin syndrome patient is caused by translational re-initiation at methionine p.26. A. The lysate of cultured keratinocytes was run on NuPAGE®Bis-Tris Pre Cast Gels System. p63α-specific antibody H-129 [Santa Cruz] was used to detect the signal on nitrocellulose membrane. This experiment demonstrates two ΔNp63α protein variants in cultured keratinocytes. In control samples (lanes 1 and 2), the smaller ΔNp63α variant is present in small quantities. However, in the Rapp-Hodgkin syndrome sample (lane 3) the ratio is about one to one. α-tubulin [Abcam] is used as a loading control. **B.** The second putative initiation site in ΔNp63, methionine p.26, was mutated to isoleucine in a wild type expression vector and transiently transfected into p63 negative Saos-2 cells, and harvested 30-48h after transfection. The cell lysates were run on 8% SDS-Polyacrylamide gel and blotted to a nitrocellulose membrane. This was incubated with p63α-specific antibody H129 [Santa Cruz]. Mock (lane 1) is negative. When transfecting the wild type ΔNp63α (lane 2) two p63 products are clearly present. However, when transfecting the mutation construct (p.M26 is mutated into isoleucine), the lower product is missing (lane 3). This indicates that methionine at position p.26 can initiate the translation. **C.** N-terminal RHS and AEC mutations were also introduced to the expression vector along with the vector containing the p.M26I mutation. These constructs were transfected into the Saos-2 cells and prepared as described above. Lane 1 contains empty vector pcDNA3. Wild type ΔNp63α (lane 2) produces the double band similar to 4B. However, when transfecting the pathogenic mutations p.Q11X (lane 3), p.Q16X (lane 5) and p.Q9fs (lane 7) the upper product is completely absent. When a combination of pathogenic mutation plus a p.M26I mutation is present (p.Q11X_M26I (lane 4), p.Q16X_M26I (lane 6) and p.Q9fs_M26I (lane 8)) no p63α protein product is detected at all. This experiment clearly demonstrates that all three pathogenic mutations cause translational re-initiation at methionine p.26.

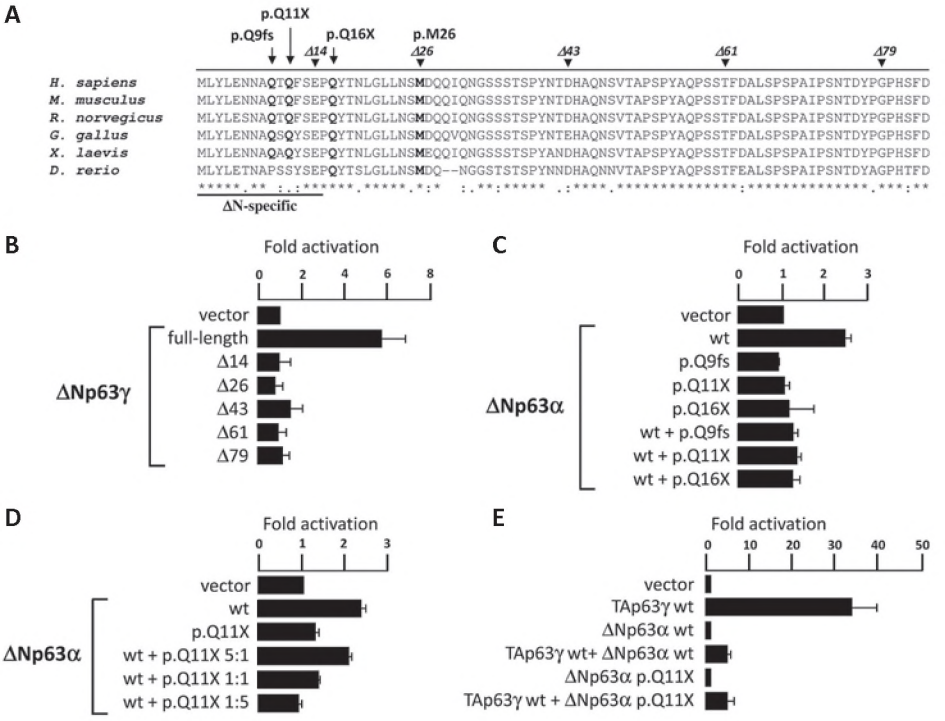

Figure 5. Transactivational activity in the N-terminus of ΔNp63 isoform. A. Multiple sequence alignment represents the conservation of N-terminus of ΔNp63: all three glutamines mutated in RHS/AEC patients and the putative second translational initiation codon p.M26 are almost fully conserved. The N-terminal truncation mutations are also indicated in this alignment. **B.** Saos-2 cells were transiently transfected with N-terminally truncated p63 cDNA expression vectors (shown in fig. 5A) and p53 promoter activation was measured. The truncated proteins lack the N-terminal 14 amino acids (Δ14 i.e. the residues which are unique for ΔNp63 isoforms) or N-terminal 26, 43, 61 or 79 amino acids (Δ26, Δ43, Δ61, Δ79, respectively). Full length ΔNp63γ can activate the p53 promoter approximately six fold, whereas deleting the first 14, 26, 43, 61 or 79 amino acids abrogates the transactivational activity completely. **C.** Saos-2 cells were transfected with empty vector (pcDNA3), ΔNp63α wild type, ΔNp63α mutant (p.Q9fs, p.Q11X and p.Q16X) or combination of wild type and mutant, and Keratin-14 promoter activation was then measured. The wild type ΔNp63α is able to activate the Keratin-14 promoter 2.5 times, whereas none of the ΔNp63α mutants is able to activate the promoter at all. Neither co-transfection of wild type and mutant are able to activate KRT14 promoter, indicating a dominant negative effect for the mutants. **D.** Saos-2 cells were transfected with wild type and p.Q11X ΔNp63α constructs in different ratios and KRT14 promoter activity was measured. 5:1 ratio of ΔNp63α wild type and p.Q11X can activate KRT14 promoter similar to ΔNp63α wild type construct, however, when using 1:1or 1:5 ratio, which mimics RHS patient situation the activation is repressed and is similar to p.Q11X construct. This supports the hypothesis that ΔΔNp63α is a dose-dependent regulator of ΔNp63α. **E.** Finally, Saos-2 cells were transfected with TAp63γ and ΔNp63α isoforms. Wild type TAp63γ can activate the p53-promoter 34 times when comparing to empty vector, whereas ΔNp63α cannot activate the promoter at all. Instead, ΔNp63α wild type inhibits the activity of TAp63γ in a co-transfection assay. This dominant negative effect against the TAp63γ is also detected in the RHS p.Q11X ΔNp63α, indicating that the mutated molecule is able to bind to the promoter.

Discussion

Until now, mutations causing RHS and AEC syndrome have only been described in exons 13 and 14, which encode the SAM and TI domains of the p63α-protein. Here we report three RHS/AEC families without any changes in these exons, but with novel pathogenic mutations in the amino-terminus of ΔNp63: p.Q9fs and p.Q11X (in the alternative exon 3') and p.Q16X (in exon 4). The first two are present in only the ΔNp63 isoforms, whereas the latter is present in both ΔNp63 and TAp63 isoforms. All these mutations lead to premature termination codons soon after the first translation initiation codon. Despite this, these mutant alleles still produce a p63-related protein by re-initiation of translation at the next ATG codon. Recently, similar translation re-initiation processes have been reported in genes causing other human inherited diseases, such as breast cancer, Menkes disease and Incontinentia pigmenti (36-39). Nevertheless, this is the first time that altered N-terminus and translation re-initiation has been linked to *p63* and RHS/AEC syndromes.

The mutations reported here are not associated with severe skin phenotype. Absence of skin defects is also observed in 60% RHS and 20% of AEC syndrome patients (28). Since we have studied only four patients, it cannot be concluded whether the lack of severe skin defects is related to the position of the mutations or just a reflection of the normal clinical variety in RHS/AEC. The three novel mutations we have reported are of particular interest, as they differ completely from the previously identified mutations in Rapp-Hodgkin and AEC syndromes. The 23 pathogenic mutations reported so far in p63 in these disorders are missense mutations, deletions or insertions clustered in the a-terminus of p63, changing the composition of the SAM and TI domains (15-29). However, our new findings, based on the sites of the p.Q11X, p.Q16X and p.Q9fs mutations, imply that ΔNp63α is the critical isoform causing RHS/AEC syndrome. One of the new mutations we report (p.Q16X/p.Q71X) also affects the TA-isoform, although since ΔNp63α is the only p63 isoform (>99%) expressed in epithelial tissues, the relevance of TA-isoform disruption remains obscure (8,40). Moreover, the phenotype of the patient carrying the p.Q16X/p.Q71X mutation is not significantly different from that of the patients with the other mutations in families 1 and 3. This strongly indicates that either an aberrant ΔN- or α-terminus of p63 cause a condition which is characterized by ectodermal dysplasia and orofacial clefting, but not developmental limb problems.

It has been shown previously that ΔNp63α can function both as an activator and a repressor of transcription (10,40,43-45). We tested transactivational activity of p.Q9fs, p.Q11X and p.Q16X in a transactivation assay on the Keratin-14 promoter, on which ΔNp63α acts as an activator of transcription. Mutant constructs are unable to activate the KRT14 promoter (Fig. 5C), indicating that the first 25 amino acids are crucial for the activation. This is in accordance with the results of transactivation assays in this paper (Fig. 5B), in which deletion mutants affect the N-terminal TA2 domain. In addition, in previous assays the N-terminal deletion abrogates the activity to transactivate p53 target genes and induce cell cycle arrest and apoptosis (10,11). Our TAp63γ co-transfection assay show that the p.Q11X mutant can inhibit TAp63γ-mediated transactivation (Fig. 5E), indicating that this mutant is able to bind to DNA and form heteromers with other p63 isoforms. Previously, we have demonstrated that the SAM domain mutant proteins have lost their ability to form p63-protein complexes and their ability to bind to DNA (18). Apparently, the ΔN-mutant proteins have retained these properties. It is unclear whether these properties have an effect on the phenotype since they are highly similar, except perhaps for the skin phenotype, which is not severe in the patients with N-terminal mutation. In addition, the ΔNp63α co-transfection assay shows that all these three mutants can inhibit ΔNp63α wild type transactivation (Fig. 5C), suggesting that these mutations have a dominant negative effect.

The first 25 amino acids in the ΔNp63α are crucial for the correct function of the ΔNp63-isoforms. The shorter ΔΔNp63α variant was also detected in wild type keratinocytes as well as in ΔNp63α-transfected Saos-2 cells (Fig. 4). In keratinocytes, this new isoform appears to be more abundant than any other p63 isoform (TA, β, γ) except ΔNp63α. This indicates that the p63 gene encodes more protein isoforms than the six that have been recognized to date (Fig. 1). Therefore, this p63 variant appears to be a novel translational variant and apparently not deleterious for the cells. We hypothesize that in keratinocytes the ΔΔNp63α isoform has a regulatory function, which is imbalanced in patients because of the increased amount of ΔΔNp63α. Our dose-dependent assay of ΔNp63α and ΔΔNp63α on KRT14 promoter also showed that elevated amount of ΔΔNp63α represses the ΔNp63α activation (Fig. 5D). Thus, the disturbed ratio between the full length and shorter p63 variant might be relevant for causing the disease phenotype. Since AEC/RHS is a dominantly inherited disease, we propose a dominant-negative effect of the ΔΔN-isoform against wild type ΔN-isoform in either a dimer or tetramer structure. Once the mutated and wild type isoform form a functional protein complex, the ΔΔN-isoform may alter the function of this complex, working as a repressor or an activator.

Both ΔN- and α-terminal mutations can lead to a highly similar clinical phenotype, although the molecular mechanisms involved in the pathogenesis are as yet unknown. The TI-domain of the α-terminus intra-molecularly binds to the N-terminal TA-domain, even though this has not been reported for the ΔN-terminus of ΔNp63α (12). It appears however, that both types of mutations have an effect on transcription regulation by ΔNp63α. Here we have shown that deletions of ΔNp63 result in reduced transactivation activity by ΔNp63α on p53 and p63 promoters. Previously we have shown that SAM-domain mutants ablate the transactivational inhibitory effects of ΔNp63α towards transactivation by p53 and TAp63γ (18). Consequently, both N-terminal and SAM-domain mutations associated with RHS/AEC syndrome appear to affect transactivation activity by ΔNp63α isoforms. A systematic screening of the effects of constructs with N- and C-terminal mutations on a panel of natural p63 target genes and in a relevant cell type, such as in keratinocytes, may give further insight on the precise disease mechanism in RHS/AEC syndrome. From the present study, we conclude that the generation of an N-terminally truncated ΔNp63α protein is responsible for causing a Rapp-Hodgkin and AEC syndrome-like phenotype.

Material and methods

Clinical study

The index patient of family 1 is a 6 year-old girl, who has a unilateral cleft lip and palate. She has coarse dry blond hair and fair skin and there is scaling on the forehead, around the nipples and on the buttocks. She is anhidrotic. At birth she had a systolic murmur, but on ultrasound was found to have a normal cardiac anatomy and the murmur appeared to be innocent. The index patient's mother also has ectodermal dysplasia: her hair has a coarse texture and a spiky appearance. Her skin is dry, and she has hypodontia of her primary teeth and her fingernails are dystrophic. She has anhidrosis on her trunk and limbs but is able to sweat normally from her palms and soles. In addition, she has had frequent urinary tract infections. Unlike her daughter she has secondary hearing loss due to a malformation of the external ear canal. She also had a history of a malignant melanoma, which had been excised from her right hand.

The index patient of family 2 is a 2 year-old boy, who was born to non-consanguineous parents of Greek-Australian descent. He has a unilateral cleft of the soft palate and left-sided ankyloblepharon filiforme. He has sparse eyebrows and eyelashes in infancy, but no alopecia or dry skin. He has small nails, which are not dystrophic. He also has hypoplastic alae nasi. There are no dental anomalies or delayed dental eruption.

The patient in family 3 is a 13 year-old girl. She was born with an absent hard palate in her mouth, ankyloblepharon filiforme and an atrial septal defect. As an infant she was prone to infections, but this later improved. She has upslanting palpebral fissures, a long nose and a small mouth with a thin upper lip. She has sparse eyelashes, but extremely thick and bushy hair, which is easy to brush. Her hands, feet and nails are all normal.

Mutation analysis

Blood samples and skin samples used in this study were obtained after written informed consent was obtained. Genomic DNA was extracted from peripheral blood samples by a standard salting-out method. All 16 exons of the *p63* gene were amplified and sequenced in both directions. Primers, which were used to amplify the alternative exon 3' and exon 4 are illustrated in Table 1A.

Cell culture

A skin biopsy (4mm diameter) was taken from the back of the index patient's mother. The skin was collected in RMPI [Gibco] medium with Gentamycin (1:1000) [Gibco], Amphotericin (1:100) [Gibco] and Penicillin/Streptomycin [Gibco]. The biopsy was then trypsinized in 0.25% Trypsin-PBS [Brunschwig] overnight at 4°C following which the upper epidermal layer was separated from the biopsy with tweezers and the dermal surface was scratched smoothly to release the keratinocytes. Serum was added to stop the trypsin activity. Next the solution containing the dermal and epidermal components was vortexed at low speed for 1 minute. The dermal parts were removed and the epidermal cells were added on to irradiated 3T3-J2 cells. Cells were cultured in Green's medium: DMEM [Gibco], Ham's F12 [Gibco] (2:1) supplemented with 10% fetal bovine serum (FBS) [Hyclone], 4 mM L-Glutamine [ICN Biomedicals], 100 U/ml penicillin [Gibco], 100 mg/ml streptomycin [Gibco], 25 mg/ml adenine [Calbiochem], 5 mg/ml insulin [Sigma], 0.4 mg/ml hydrocortisone [Calbiochem], 1,4 ng/ml triiodothyronine [Sigma], 0.1 nM Cholera toxin [Sigma]. Epidermal growth factor (EGF) (10 ng/ml) [Sigma] was added to the medium three days after starting the keratinocyte culture. The medium was refreshed every second day until the culture reached 80-90% confluence. The primary keratinocyte stocks were stored in liquid nitrogen.

Mouse fibroblast cell line 3T3-J2 was used as a feeder layer for the keratinocytes in Green's medium. Pure 3T3-J2 cells were cultured in DMEM w/o pyruvate [Gibco] containing 10% calf serum supplemented by iron [Hyclone] and 100 U/ml penicillin [Gibco] and 100 µg/ml streptomycin [Gibco]. 3T3 cells were irradiated at 3500 cGy about 20 hours before adding keratinocytes.

Keratinocytes were cultured at 37°C in 5% CO2 in keratinocyte growth medium. This medium consists of Keratinocyte Basal Medium, 0.15mM Ca2+ [Cambrex], supplemented with 0.1mM ethanolamine [Sigma], 0.1mM phosphoethanolamine [Sigma], bovine pituitary extract (0.4%) [Bio Whittaker], 10ng/ml EGF [Sigma], 5mg/ml insulin [Sigma], 0.5µg/ml hydrocortisone [Calbio-

Table 1. Oligonucleotide sequences.

A	GenBank number	Exon	Forward (5' > 3')	Reverse (5' > 3')	Primer for
	NT_005612.15	*p63* Exon 3'	gcctcctcatgcctatagttg	tcttacagccaccacagaaaa	PCR, sequencing
	NT_005612.15	*p63* Exon 4	gatccgtggcttcagcgg	aagcccatccttggacttgg	PCR, sequencing

B	GenBank number	Isoform	Forward (5' > 3')	Reverse (5' > 3')	Primer for
	AF_075431	ΔNp63	ctggaaaacaatgcccagac	gggtgatggagagagagcat	RT-PCR

C	GenBank number	Isoform/gene	Forward (5' > 3')	Reverse (5' > 3')	Primer for
	AF_075431	ΔNp63	caatgcccagactcaatttagtga	tgctggtccatgctgttcag	RT-qPCR
	AF_075431	ΔNp63	ttgtacctggaaaacaatgcc	tgctggtccatgctgttcag	RT-qPCR
	AF_075431	p63α	tccatggatgatctggcaagt	gcccttccagatcgcatgt	RT-qPCR
	NM_001101	ACTB	actggaacggtgaaggtgaca	agggacttcctgtaacaacgca	RT-qPCR
	NM_001002	hARP	caccattgaaatcctgagtgatgt	tgacaagcccaaaggagaag	RT-qPCR
	NM_002046	GAPDH	tgcaccaccaactgcttagc	ggcatggactgtggtcatgag	RT-qPCR

D	Plasmid	Change	Forward (5' > 3')	Reverse (5' > 3')	Primer for
	pcDNA3_Mm_ΔNp63α	p.Q11X (c.31C>T)	gaaaacaatgcccagacttaatttagtgagccacagt	actgtggctcactaaattaagtctgggcattgttttc	Mutagenesis
	pcDNA3_Mm_ΔNp63α	p.Q16X (c.46C>T)	caatttagtgagccatagtacacgaacctgg	ccaggttcgtgtactatggctcactaaattg	Mutagenesis
	pcDNA3_Mm_ΔNp63α	p.Q9Xfs (c.26delA)	ctggaaaacaatgcccgactcaatttagtgag	ctcactaaattgagtcgggcattgttttccag	Mutagenesis
	pcDNA3_Mm_ΔNp63α	p.M26I (c.78G>A)	gctcctgaacagcatagaccagcagattcag	ctgaatctgctggtctatgctgttcaggagc	Mutagenesis
	pcDNA3_Mm_ΔNp63γ	Δ14	ccacagtacacgaacctggggctcc	catccgcggggcaggggtcccggaa	Mutagenesis
	pcDNA3_Mm_ΔNp63γ	Δ26	gaccagcagattcagaacggctcc	catccgcggggcaggggtcccggaa	Mutagenesis
	pcDNA3_Mm_ΔNp63γ	Δ43	cacgcacagaatagcgtgacggcgc	catccgcggggcaggggtcccggaa	Mutagenesis
	pcDNA3_Mm_ΔNp63γ	Δ61	tttgatgccctctctccatcccctg	catccgcggggcaggggtcccggaa	Mutagenesis
	pcDNA3_Mm_ΔNp63γ	Δ79	cacagcttcgatgtgtccttccagc	catccgcggggcaggggtcccggaa	Mutagenesis

chem], 100U/ml penicillin [Gibco] and 100µg/ml streptomycin [Gibco]. The medium was changed every second day until the culture reached confluent state.

Transfection conditions

Human osteoblast cell line Saos-2 was used for transient transfections. Saos-2 cells were cultured in DMEM [Gibco] supplemented by 10% fetal calf serum [Sigma], 1% natrium-puruvate [Gibco], 1% Glutamax-1 [Gibco] and 100 U/ml penicillin [Gibco] and 100 µg/ml streptomycin [Gibco]. Approximately 1.5 x 105 Saos-2 cells were seeded in one well of a 6-well tissue culture plate. Effectene transfection reagent [Qiagen] was used to transfect the pcDNA3, pcDNA3_Mm_ΔNp63α (43) wild type and its mutant versions p.Q11X, p.Q16X, p.Q9fs, p.M26I and double mutant p.Q11X_M26I, p.Q16X_M26I and p.Q9fs_M26I constructs into Saos-2 cells. The same method was used for co-transfections, where either pcDNA3_ΔNp63α wild type was transfected together with each single mutant (p.Q11X, p.Q16X, p.Q9fs) ΔNp63α construct or pcDNA3_TAp63γ wild type was transfected together either with wild type or mutant (p.Q11X) pcDNA3_ΔNp63α construct. The cells were collected 30-48 hours after transfection.

For transfections of the ΔNp63 truncation mutations (Δ14, Δ26, Δ43, Δ61, Δ79), Saos-2 cells were plated on 18 mm round glass coverslips and a total of 2 µg plasmid DNA was transfected using a Calcium phosphate precipitation protocol (46).

Luciferase assays

Subconfluent Saos-2 cells were transfected as described above at a 1:2 ratio between the reporter construct (firefly luciferase under control of Keratin-14 promoter) (kind gift of Dr B. Andersen and Dr E. Candi) and the wild type or mutant (p.Q11X, p.Q16X, p.Q9fs) pcDNA3_ΔNp63α or a combination of wild type and mutant vector. In addition, 50ng of Renilla luciferase construct was co-transfected in each transfection to normalize for transfection efficiency. After 30 hours the transfection luciferase activities were measured using a Dual Luciferase Reporter Assay System [Promega] according to the manufacturer's instructions. The level of activation was calculated in comparison to transfections with an empty pcDNA3 vector.

Transfections for the TAp63γco-transfection assay were carried out as described above at a 1:3 ratio between the reporter construct (β-galactosidase reporter under p53 promoter) and expression construct (pcDNA3_Mm_TAp63γ wild type, pcDNA3_ΔNp63α wild type or p.Q11X mutant construct, or in co-transfections TAp63γ in combination with ΔNp63α wt or mutant). In addition, 5ng of Renilla luciferase construct was transfected in each transfection to normalize for transfection efficiency. After 30 hours the transfection β-galactosidase and luciferase activities were measured using a Luminescent beta-galactosidase detection kit II [Clontech] and a Renilla Luciferase Reporter Assay System [Promega] according to the manufacturer's instructions. The level of activation was calculated in comparison to transfections with an empty pcDNA3 vector. Transactivation assays of the truncation mutations were carried out as described previously (43).

Mutagenesis

Pathogenic mutations of interest were introduced into the pcDNA3_ΔNp63α vector by using QuickChange Site-Directed Mutagenesis kit [Stratagene] according to the manufacturer's instructions. The p.Q11X, p.Q16X, p.Q9fs and p.M26I mutations were introduced in this vector. The p.Q11X, p.Q16X and p.Q9fs mutations were also introduced into pcDNA3_ΔNp63α_p.M26I mutation construct to create double mutations. Mutagenesis primers are shown in Table 1D. The correct sequence of each clone was determined by direct sequencing of the entire cDNA insert.

N-terminal deletion mutations were introduced into pcDNA3_Mm_ΔNp63γ vector by using ExSite PCR-based site-directed mutagenesis procedure [Stratagene] according to the manufacturer's instructions. The sequences encoding amino acids 2-14, 2-26, 2-43, 2-61 and 2-79 (amino acid numbers referring to ΔNp63 protein) were removed using oligonucleotides described in Table 1D. The obtained clones were screened for the presence of the respective deletions by direct sequencing using primers flanking the deletions.

Sample preparation and immunoblotting

Transfected Saos-2 cells and keratinocytes were harvested in PBS and centrifuged at 4°C at 3000 RPM for 10 minutes. The pellet was lysed in lysis buffer containing: 50mM Tris-HCl pH 7.8, 10% glycerol [Invitrogen], 0.5% Nonidet-P40 [Brunschwig], and 5mM Ethylene glycol-bis-tetraacetic acid (EGTA) [Fluka], and freshly added 10mM β-mercaptoethanol [Sigma], 0.5mM Phenylmethanesulfonyl fluoride (PMSF) [Fluka], 1 µg/ml Pepstatin A [Fluka] and 1x Protease inhibitory cocktail [Roche], for 20 minutes on ice. Immunoblotting was performed using the NuPAGE®Bis-Tris Pre Cast Gel System [Invitrogen] following the manufacturer's instructions. Samples were run on a 4-12% NuPAGE Bis-Tris gel in MOPS buffer [Invitrogen]. p63α-specific polyclonal antibody H-129 (1:500) [Santa Gruz] was used to detect p63 and a mouse monoclonal α-Tubulin antibody DM1A (1:5000) [Abcam] was used as a loading control. Alexa-680 goat-anti-rabbit secondary antibody [Molecular Probes] and IrDye-800 goat-anti-mouse secondary antibody [Rockland] were both used in 1:5000 dilution. The signal detection was performed by Odyssey scanner [Licor].

RNA isolation

Keratinocytes were cultured in Keratinocyte growth medium (KGM) and harvested either at confluent state or after 48h differentiation. Total RNA isolation was performed using the RNeasy mini kit [Qiagen] according the manufacturer's instructions. RNA was treated with DNase I while bound to the RNeasy column to remove residual traces of genomic DNA [Qiagen]. The integrity of the RNA was assessed on an agarose gel, and the concentration and purity were determined with a ND-1000 spectrophotometer [Nanodrop].

Reverse transcriptase PCR

Two µg of total RNA was transcribed into cDNA as described earlier (47). cDNA was amplified with a forward primer specific to alternative exon 3' of *p63* gene and a reverse primer specific to exon 4 with a total number of 35 cycles. Primer sequences are in Table 1B. The RT-PCR product was

electrophoresed in an agarose gel and purified using the Qiaquick gel extraction kit [Qiagen]. This cDNA product was sequenced with reverse primer by using a 3730 DNA Analyzer from Applied Biosystems.

Quantitative PCR (qPCR)

Quantitative PCR was performed on the iQ-apparatus (MyiQ single-color real-time detection system [Biorad]) by using iQ SYBR®Green Supermix [Biorad] according to the manufacturer's protocol. All primer pairs were designed such that they cover either separate exons or that one is spanning an exon-exon boundary. All primers were validated in triplicate by use of serial cDNA dilutions, and were confirmed for 100% ± 5% efficiency. Differences in the expression of a gene of interest between two samples were calculated by $2^{\Delta\Delta Ct}$ method (48,49). To normalize the amount of cDNA we used three housekeeping genes: *beta-Actin (ACTB)*, *hARP (human Acidic ribosomal protein)* and *GAPDH (Glyceraldehyde-3-phosphate dehydrogenase protein)*. All samples were used in duplicate and housekeeping genes were run on the same plate in the iQ-apparatus as the gene of interest. Primer sequences are provided in Table 1C.

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Web Resources

Accession numbers and URLs for data presented in this article are as follows:

GenBank, http://ncbi.nlm.nih.gov/GenBank (Accession numbers NT_005612.15, AF_075430, AF_075431, NM_000526, NM_001101, NM_001002 and NM_002046 (These are in Table 1), AAG45610, AAP87985, CAC37099, BAB20631, AAK15622, AAN03691 (these are in multiple sequence alignment in Fig. 5A).

Online Mendelian Inheritance in Man (OMIM), http://ncbi.nlm.nih.gov/Omim/.

Translation Start Prediction Netstart 1.0, http://www.cbs.dtu.dk/services/NetStart-1.0.

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Genome-wide profiling of p63 binding sites identifies genes and regulatory elements for p63-related disorders: Elucidation of the genetic basis of Split hand/foot malformation type 1

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Abstract

Heterozygous mutations in the *p63* gene are associated with phenotypically related disorders comprising ectodermal abnormalities and limb defects. We identified p63 target genes and binding sites responsible for ectodermal defects by genome-wide profiling of p63 binding using ChIP-seq and expression analysis in human primary keratinocytes from patients with *p63* mutations. In a patient with Split hand/foot malformation type 1 (SHFM1), we identified a novel de novo microdeletion that includes a p63 binding site functioning as a *cis*-regulatory element to control expression of the distally located *DLX5/DLX6* genes essential for limb development. Our data demonstrate that target genes and regulatory elements detected in this study can serve as powerful tools to identify causative mutations of unresolved ectodermal disorders.

Introduction

Transcription factor p63, a member of the p53 family, is a master regulator of ectodermal development. The key function of p63 in ectodermal development is underscored by phenotypic features in *p63* knockout mice (1,2) and by *p63* knock-down zebrafish (3). The developmental defects in animal models are reminiscent of those in p63-associated human disorders. Heterozygous mutations in *p63* give rise to at least seven dominantly inherited clinical conditions with three major characteristics, split hand/foot malformation (SHFM), orofacial clefting (OFC) and ectodermal dysplasia (ED) which manifests as defects in skin, hair, teeth, nails and exocrine glands (4,5). The most prominent of these disorders is the EEC syndrome which combines all of the three phenotypic hallmarks and is almost invariably caused by missense mutations in the DNA binding domain of p63 (6). Mutations of *p63* have been found in only a minority of other disorders, such as in isolated cleft lip/palate (0.1%) and in isolated SHFM4 (10%) (6). In addition, there is a large group of ectodermal dysplasia syndromes with phenotypic defects that resemble *p63*-associated syndromes (7). The genetic basis of many of these *p63*-related conditions, referred to as the *p63* phenotype network, is presently unknown. There is ample evidence that diseases clustered within such phenotype networks are caused by mutations in functionally related genes that constitute a gene network (8). Elucidation of functional interactions among genes within the *p63* gene network, their encoded proteins and regulatory elements which control expression of these genes may reveal new candidate genes for genetic disorders from the *p63* phenotype network.

Identifying the disease-related p63 target genes is an important step to dissect the *p63* gene network. Previous studies have focused on individual genes based on their known functions, expression patterns or related disease phenotypes. Proximal promoter regions of these genes were tested for p63 binding and their regulation by p63. These approaches resulted in a number of direct target genes of p63, such as *Dystonin* (9), *KRT14* (10), *DLX5* and *DLX6* (11). Several recent studies aimed to identify targets of p63 on genome wide scales using either expression array or Chromatin Immuno-precipitation (ChIP) in combination with DNA array analysis (ChIP-on-chip) (12-16). However, due to different cellular systems and techniques, overlap of the reported target genes is very small or difficult to determine. Moreover, none of these studies used physiological human material, and therefore contribution of the reported target genes to the phenotypes of p63-linked patients remains unclear. Furthermore, transcriptional regulation of p63 target genes by short- and long-range regulatory elements has not been investigated and could be of importance, as regulatory elements have been shown to control spatial-temporal

expression of developmental genes (17-19). We have therefore integrated genome-wide profiling approaches in a physiologically relevant disease model to resolve the p63 disease-related regulatory network.

Skin is prominently affected in p63-associated disorders, and ΔNp63α, the most common isoform of p63, is highly expressed in the basal layer of epidermis that consists mainly of keratinocytes. We established human primary keratinocyte cultures (HKCs) from control individuals and patients with *p63* mutations as our major model system to elucidate p63 disease networks under physiological conditions. Using a combination of genome-wide DNA-binding profiling by ChIP followed by deep sequencing (ChIP-seq) and expression array analysis in primary keratinocytes from EEC patients, our work is the first systematic study to search for target genes and regulatory elements of p63 that are relevant to the pathology of p63-associated and clinically related disorders. We show the proof of principle of this approach by identifying the loss of a p63 binding site within a cis-regulatory element for *DLX5/6* genes essential in limb development in an SHFM1 patient. Our approach has yielded a highly informative catalogue of p63 target genes and regulatory elements comprising the *p63* gene network, which will serve the genetic research community in the identification of causative mutations in related developmental disorders where the causative genes are yet to be identified.

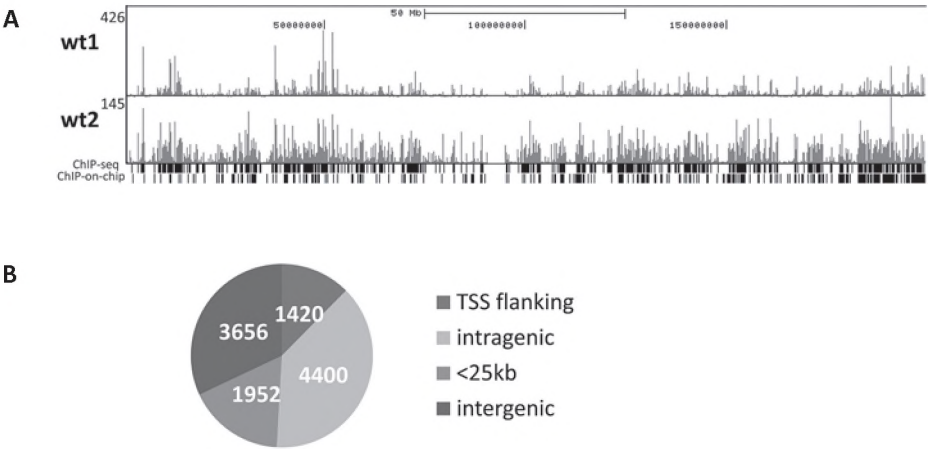


Figure 1. Characterization of identified p63 binding sites. A. A screenshot of chromosome 3 using UCSC genome browser shows similar DNA-binding profiles from our ChIP-seq analysis of two normal human primary keratinocyte cell lines (wt1 and wt2). The p63 binding sites analyzed with MACS (20) using *P* value $1x10^{-9}$ are shown in the row 'ChIP-seq', and previously reported p63 binding sites in ChIP-on-chip analysis (14) are shown in the row 'ChIP-on-chip'. **B.** Distribution of the p63-binding site location relative to RefSeq genes. Locations of binding sites are divided into: TSS flanking region (5 kb upstream of TSS, first exon and first intron), intragenic region (all introns and exons except first), < 25 kb (5–25 kb upstream or 25 kb downstream of last exon), or intergenic regions (everything else).

Results

Genome-wide p63 binding profile in human primary keratinocytes

To identify direct target genes of p63 in a physiologically relevant cell system, we obtained high-resolution global binding profiles of p63 from two independent control HKC cell lines established from unrelated control individuals (wt1 and wt2) using ChIP-seq.Analysis of the sequenced reads in these two profiles using peak recognition algorithm of Model-based Analysis of ChIP-Seq (MACS) (20) with a strict *P* value threshold (10^{-9}) gave a highly significant overlap of 11,425 peaks (*P* < 1E-300) (Fig. 1A). We considered the overlapping 11,425 peaks as trustworthy binding sites of p63 in HKCs, and continued with further analysis. From a selection of 17 binding sites of various peak heights (11-286 reads), we unambiguously validated 16 sites by ChIP followed by qPCR analysis (ChIP-qPCR) with two antibodies recognizing different epitopes of the p63 protein (Supplementary Table 1, Supplementary Fig. 1), showing that the obtained binding profile is highly reliable. Among identified binding sites, 2468 are present in the 5807 binding sites (40%) reported previously using the ChIP-on-chip technique in a cervical carcinoma cell line (14). Considering different cell types and techniques used in the DNA-binding profiling, the overlap of these two studies is remarkable. Moreover, our ChIP-seq analysis appears to be more sensitive to detect binding sites and has a higher resolution with a median peak width of 356 bp (Supplementary Table 1) compared to 1467 bp in ChIP-on-chip (14).

More than 1,400 out of 11,425 detected binding sites overlapped with transcription start site (TSS) flanking regions between 5kb upstream of the TSS and the end of the first intron of genes, which is highly significant compared to random distributions (*P* < 0.001) (Fig. 1B). In total, 10,852 genes have one or more p63 binding sites within 25 kb up- and down-stream. GO annotation of these 10,852 genes using DAVID (21) showed enriched functional categories, mainly in development but also in other biological processes such as intracellular signaling cascade, cell adhesion and cell death (Supplementary Table 2).

To assess the motifs in the detected binding sites, we applied *a de novo motif* prediction pipeline (see Material and methods for details). We identified a highly significant motif (Supplementary Fig. 2A, Supplementary Table 3), which is similar to the reported p63 and p53 motifs (Supplementary Fig. 2A-E) (14,15,22,23). We combined our previously developed p53scan algorithm (23) with the newly identified p63 Positional Weight Matrix (PWM), hereafter referred to as p63scan. We compared the p63scan to the previously described motif algorithms p63MH (22) and p53scan (23) using a Receiver Operator Curve (ROC). The performance of our p63scan showed clearly a higher sensitivity for motif recognition without compromising the specificity (Supplementary Fig. 3F). Using p63scan, we found that 10,659 out of the total 11,425 detected binding sites (93%) contain at least one p63 motif (False Discovery Rate, FDR 10%), indicating that the identified binding sites in this study are highly specific for direct binding of p63.

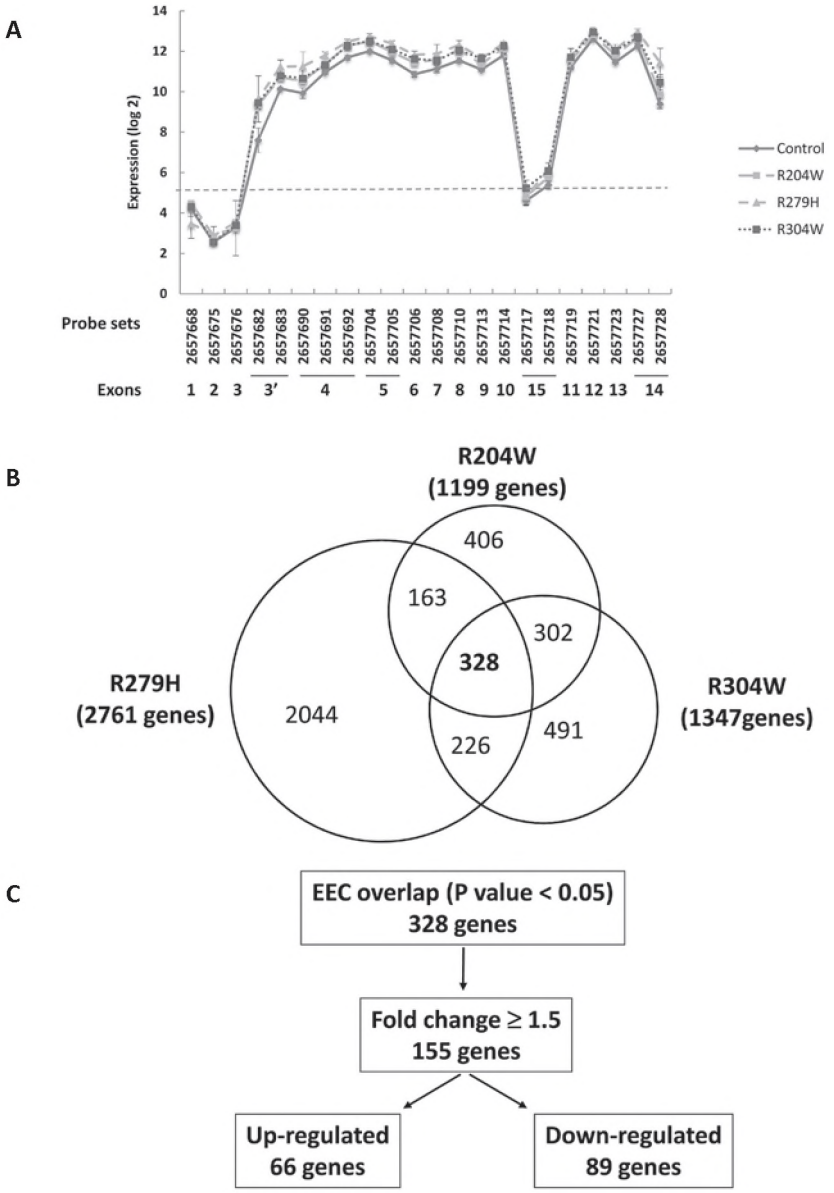


Figure 2. Expression analysis of normal human primary keratinocyte lines and patient cell lines carrying EEC mutations. A. Expression of each individual core probe sets (Supplementary Fig. 4) shows that expression of exon 3' and exons 4-14, which correspond to the ΔNp63α isoform, were at least 105-fold higher than exons 1-3 and exon 15. **B.** Overlap of differentially expressed genes in 3 EEC patient cell lines. Expression analysis of 5 independent normal human primary keratinocyte lines and 3 patient lines carrying EEC mutations, p.R204W, p.R304W or p.R279H, was performed on Affymetrix Human Exon 1.0 ST Array, and differentially expressed genes were analyzed using ANOVA model (P value < 0.05). In total, these patients shared 328 genes that were differentially expressed when comparing to the control group. **C.** Out of 328 genes, 155 showed consistent expression change more than 1.5-fold. Among them, 89 of the genes were down-regulated, and 66 genes were up-regulated in EEC patients.

Differentially regulated genes in keratinocytes from normal individuals and EEC patients

To identify genes regulated by p63, we studied genome-wide expression profiles by using Affymetrix Human Exon 1.0 ST arrays in our disease model, HKCs established from three unrelated EEC syndrome patients carrying p.R204W, p.R279H and p.R304 W mutations and five control individuals (for clinical details see Supplementary Table 4). This array utilized probe sets representing each exon (Supplementary Fig. 4B and 4C), allowing expression analysis of different isoforms. The results clearly demonstrated that ΔNp63α is the only isoform expressed at a high level in HKCs (Fig. 2A, Supplementary Fig. 4).

Hierarchical clustering analysis showed that gene expression in five normal control samples was similar and clustered away from that in three mutants p.R204W, p.R304W and p.R279H (Supplementary Fig. 5). We applied an ANOVA model using a *P* value threshold of 0.05 to identify differentially expressed genes. We identified 328 genes that showed significant and consistent changes in the same direction among all three EEC patients compared to the controls, henceforth referred as differentially expressed genes (Fig. 2B, Supplementary Table 5). Among these genes, 155 genes (89 up- and 66 down-regulated) showed fold changes higher than 1.5-fold (referred to as genes with 1.5-fold change) (Fig. 2C, Supplementary data). With independent RT-qPCR analysis, we validated 16 out of 20 genes with the highest differential expression or which were known to associate with *p63*-resembling phenotypes (Supplementary Table 7 and 8). In GO annotation analysis of 328 differentially expressed genes using DAVID (21), specific development-related GO terms such as ectodermal development and morphogenesis of an epithelium were shown to be the most significant (Supplementary Table 9).

To identify direct target genes of p63 that are abnormally regulated by mutant p63 in EEC patients, we reasoned that genes with changed expression and with p63 binding sites nearby are the best candidates of this subgroup of p63 targets. Therefore, we examined how many differentially expressed genes in our array analysis are in the vicinity of a p63 binding site. We found that 198 out of 328 differentially expressed genes (60%) have p63-binding sites in the gene or in the region within 25 kb of the gene (Table 1, Supplementary data), which represents an enrichment of 2.3-fold for all genes with binding sites (*P* = 9.31E-19). Compared to the 10-20% of the regulated genes that contain p63 binding sites reported previously (14), our DNA-binding and expression analyses were more effective in predicting direct target genes of p63. GO annotation of these 198 genes revealed similar development-related terms to that from our expression analysis (Supplementary Table 11). We considered these 198 genes as potential direct target genes of p63.

Table 1. Over-representation of genes with p63 binding sites among genes with significant changes in patient keratinocytes.

Gene groups	Total genes	Genes with binding sites[a]	*P* value[b]
A: With significant changes (P value < 0.05)	328	198	9.31E-19
B: A + Fold change > 1.5 fold	155	92	1.49E-08
C: B + Consistently down-regulated	66	49	3.50E-10
D: B + Consistently up-regulated	89	43	0.0168

[a] Genes with binding sites are genes that have at least one p63 binding site either in the gene or within 25 kb genomic region of the gene. [b] Hypergeometric analysis.

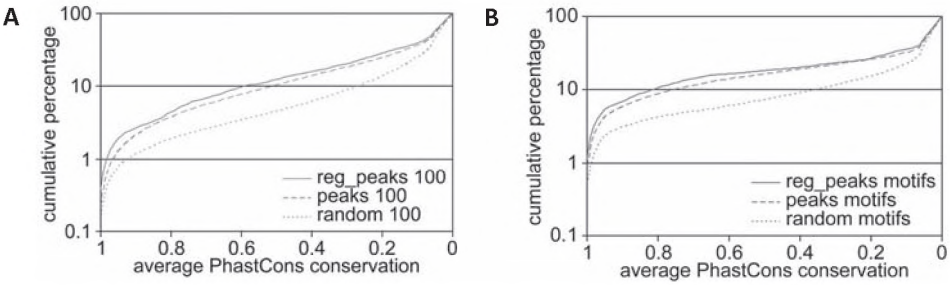


Figure 3. Conservation of p63 binding sites and motifs in vertebrates. The percentage of p63 binding sites (y-axis) is shown for decreasing PhastCons conservation scores (x-axis). **A.** The average PhastCons conservation score of 100-nucleotide regions centered at the summit of 11,425 p63 binding peaks (peaks 100), of 198 peaks located near 328 differentially regulated genes in three patients (reg_peaks 100), and of 100,000 regions randomly chosen from the whole genome (random 100). **B.** The average PhastCons conservation score of 10nt p63 motifs from 10,675 motifs in the p63 peaks detected in the genome (peaks motifs), from 294 motifs near differentially regulated genes (reg_peaks motifs), and from 7,600 motifs found in random genomic regions (random motifs). For a color figure see Appendix 2.

Subsequently we investigated the number of genes with binding sites in 155 genes with 1.5-fold change including both 89 up- and 66-down regulated genes (Table 1), and asked whether differences in p63 motifs can lead to different regulation by p63. By searching for p63 motifs in the binding sites near genes, we found that there are typical p63 motifs in binding sites of both up- and down-regulated genes and there is no major difference between them (Supplementary Fig. 6). These data are consistent with gene activation or repression being determined by other co-factors rather than motif recognition by p63.

Evolutionary conservation of p63 binding sites

As a member of the *p53* gene family showing evolutionary divergence in vertebrates, *p63* has a single copy in the vertebrate genomes (24). We therefore assessed the evolutionary conservation of the identified p63 binding sites and p63 motifs in these binding sites in vertebrates.
The identified binding sites are significantly more conserved than random sequences of the same size. Remarkably, the conservation scores of all binding sites are only slightly lower than that of the binding sites near the differentially expressed genes (Fig. 4). This suggests that p63 binding sites which are not close to differentially regulated genes are also functionally relevant. Similarly, p63 motifs in identified binding sites are more conserved than random expectation and the conservation conservation scores from p63 motifs are higher than those from the binding sites (Fig. 3A and 3B).

Transcriptional regulation by p63 through identified binding sites

To test whether p63 regulates transcription through the identified binding sites, we cloned genomic regions containing p63 binding sites near or in the interesting potential targets in a luciferase reporter construct and tested their transactivation activities in transient transfection assays in Saos2 cells. Out of total 14 tested binding sites, 12 binding sites were shown responsive to p63.

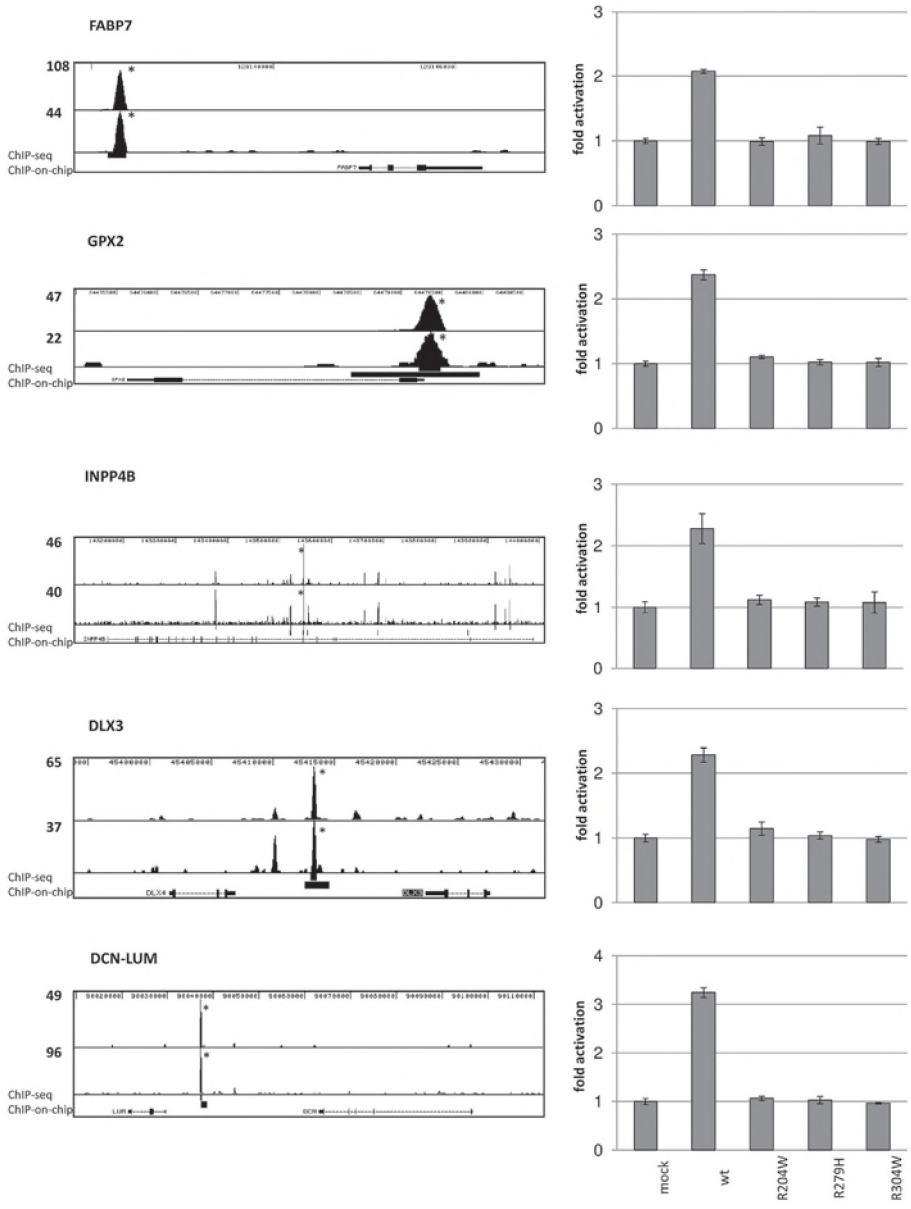
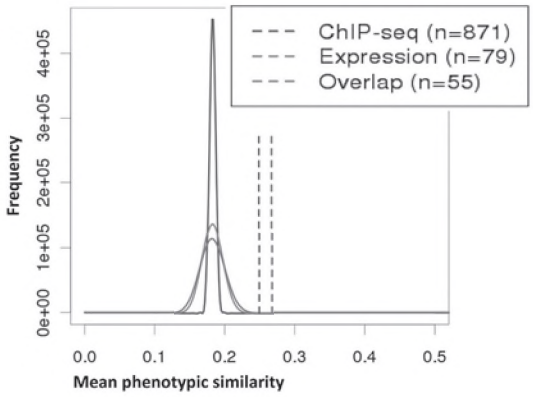


Figure 4. Reporter assays on several identified p63 binding sites in ChIP-seq analysis. Left panel, screenshot from UCSC genome browser of p63 binding peaks in two independent human primary keratinocyte cell lines (wt1 and wt2). The p63 binding sites analyzed with MACS (20) are shown in the row 'ChIP-seq', and previously reported p63 binding sites in ChIP-on-chip analysis (14) are shown in the row 'ChIP-on-chip'. The asterisk represents the binding sites that were tested in transfection assays. Right panel, indicated binding sites of p63 were cloned in a luciferase reporter construct and tested for their ability to regulate transcription in Saos2 cells. For all shown constructs, ΔNp63α activates transcription through the tested binding sites and EEC mutations in ΔNp63α abolish transactivation. Transactivation through the binding site upstream of the *IRF6* gene is shown elsewhere (manuscript under review).

A



B

Feature description	P-value
Epithelium	8,34E-11
Adhesion	2,54E-09
Extracellular Matrices	1,32E-08
Basal Lamina	1,38E-07
Membrane	6,28E-07
Corneal Stroma	1,64E-06
Cell, Epithelial	3,19E-06
Dentition	4,26E-06
Extracellular Space	1,14E-05
Hyalin	1,48E-05

Figure 5. Association of p63 potential target genes with diseases. A. Genes identified in ChIP-seq and expression analyses and the 198 potential direct target genes (overlap, differentially expressed genes with binding sites) were examined using OMIM disease base to determine their associated diseases (871, 79 and 55 OMIM IDs, respectively) and the affected feature terms in these diseases. The similarity scores from diseases associated with each group of genes are indicated with dashed lines. Diseases associated with genes identified in expression analysis (red) and the potential direct target genes (overlap, purple) showed very similar affected features and are not distinguishable from each other. Diseases linked to genes identified from ChIP-seq analysis (blue) also showed significantly similar features. The same number of diseases associated with each group of genes are randomly selected from the OMIM database. The distribution of the similarity scores from these randomized diseases are calculated and shown in solid lines. **B.** Over-represented feature descriptions in diseases associated with 198 potential target genes. For a color figure see Appendix 2.

Binding sites in or near the *FABP7, GPX2, INPP4B, DLX3, DCN-LUM* and *IRF6* genes are responsive to p63, as wild type p63 activates transcription through these binding sites. In contrast, the EEC syndrome mutations p.R204W, p.R279H and p.R304W disrupted the transactivation activity (Fig. 4 and data not shown). These data are consistent with p63 binding to the identified binding sites to regulate transcription, and that EEC mutations disrupt DNA-binding of p63 and affect transcriptional regulation.

Association of p63 gene network with disease phenotypes

Having established a list of p63 target genes, we investigated whether these genes contribute to patient phenotypes. An OMIM database search for diseases associated with identified target genes identified 32 out of 198 potential direct target genes which have already been associated with diseases with similar features to p63-related disorders. Examples include *TGFBI*/corneal dystrophy, *IRF6*/orofacial clefting, *DFNA5*/deafness and *LAMB3*/skin disease (Supplementary data). Most of these phenotypic features were seen in all three EEC syndrome patients from whom the HKCs were derived. In total, 871 and 79 OMIM disease IDs are associated with genes identified from ChIP-seq and expression analyses, respectively, and 55 disease IDs are associated with the 198 potential direct targets (Fig. 5A, Supplementary data). To assess how these diseases are related to each other, we looked for the affected features in these diseases and analyzed their similarities (Fig. 5A and 5B).

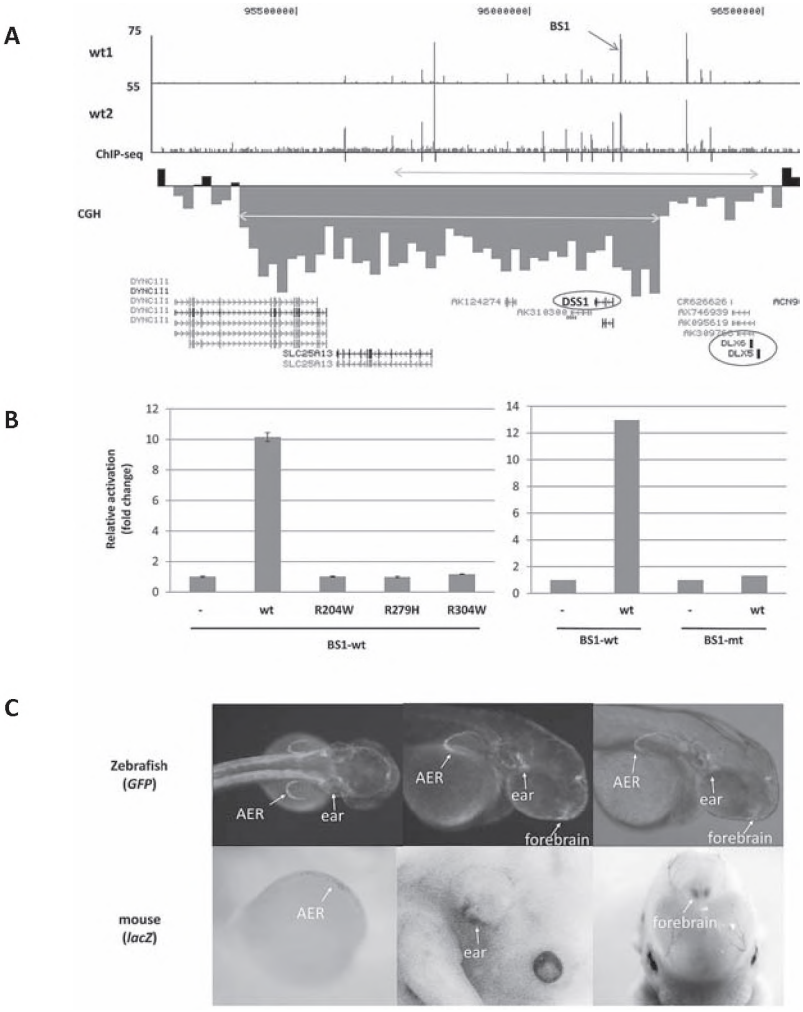


Figure 6. Function analysis of p63 binding sites at SHFM1 locus on chromosome 7. A. A screenshot of UCSC genome browser shows the p63 binding profile from two HKC cell lines (wt1 and wt2) and several strong binding sites were identified near *DLX5* and *DLX6* on chromosome 7. Ultra-high Comparative Genomic Hybridization array analysis performed with DNA of a SHFM1 patient showed a chromosomal deletion of 880 kb on chromosome 7 (95,390,000-96,270,000, hg18, the orange arrow) which includes *DSS1*, *SLC25A13* and part of *DYNC1I1* but not *DLX5* and *DLX6*. Breakpoints and 20kb averaged log2 ratios were visualized in the genome browser. A previously reported minimal deletion determined by markers D7S527 and D7S1796 (30) is marked with a green arrow. **B.** The p63 binding site SHFM1-BS1 was tested in transient transfection assays. Transcription of the luciferase reporter gene was strongly activated by ΔNp63α through SHFM1-BS1 binding site and the activation was abolished by **p63** EEC mutations p.R204W, p.R279H and p.R304W (left panel). Activation was also abolished when point mutations were introduced into the p63 binding motif in the SHFM1-BS1 binding site (right panel). **C.** SHFM1-BS1 was cloned in reporter constructs carrying the GFP or the LacZ gene to generate transgenic zebrafish and mice, respectively. Expression of the GFP gene in zebrafish and the LacZ gene in mice showed that SHFM1-BS1 can control gene expression at the apical ectodermal ridge (AER), ear and forebrain. The red fluorescence expressed in the muscles in the first zebrafish panel corresponds to the positive control of transgenesis.

The similarity scores (0.27) of feature descriptions associated with differentially expressed genes (expression) and with 198 potential direct targets (overlap) are significantly higher than random expectation ($P < 1E-5$) and not distinguishable from each other (Fig. 5A). Interestingly, the similarity score for the features derived from genes mapped by ChIP-seq binding sites is also significantly higher than random distribution ($P < 1E-5$) (Fig. 5A). The significant similarity of features extracted from DNA-binding profile suggests that the majority of the identified binding sites are relevant to developmental defects. Indeed, the over-represented feature descriptions of these diseases are similar to those of EEC syndrome caused by p63 mutations: epithelium, adhesion and extracellular matrices (Fig. 5B). Thus, the identified direct target genes of p63 are likely to be responsible for the phenotypes of p63-related diseases, the p63 phenotype network.

Regulatory elements to control gene expression in orofacial development

Our ChIP-seq and expression analyses revealed a large difference in the number of regulated genes (10,852 versus 328). One explanation for this is that some of the binding sites function as regulatory elements to control gene expression in other processes than proliferation and differentiation of adult keratinocytes. We hypothesized that a proportion of the binding sites have a role in tissue- or development-specific transcription regulatio n. Therefore, we examined a common feature of p63-associated disorders, cleft lip/palate, which could result from abnormal gene expression in early development. In the databases of human diseases (POSSUM database) and in mouse models (Mouse Genome Informatics, The Jackson lab), 358 genes are found to be associated in cleft lip/palate (Supplementary data). Among them there are 145 genes with p63 binding sites, which represent an enrichment over genes with binding sites in the entire genome ($P = 7.29E-8$). Most of these genes (347 out of 358 genes) do not show differential expression change in our expression analysis of HKCs (Supplementary data). One notable exception to this is the *IRF6* gene, for which we showed that p63 activates transcription through a binding site located 10-kb upstream of the transcription initiation site (manuscript under review). Interestingly, IRF6 has an important role in skin as well as in palatal development and heterozygous *IRF6* mutations are associated with developmental defects in both organ structures (25,26). Hence, our data strongly indicate that p63 regulates gene expression at a certain stage of development and disruption of expression may lead to developmental defects.

A microdeletion in SHFM1 includes a p63 binding site acting as a cis-regulatory element

In addition to orofacial development, we assessed the role of p63 binding sites in human limb abnormalities. Split hand/foot malformation (SHFM) is one of the common features in p63-related patients. Genes have been associated with SHFM, including *p63* on chromosome 3 (SHFM4) (4) and Dactylin on chromosome 10 (SHFM3) (27). The SHFM1 locus on chromosome 7 contains *DLX5* and *DLX6* which have been shown as target genes of p63 and are co-expressed with p63 in the apical ectodermal ridge (AER) of the developing limbs (11). In mice, simultaneous deletion of both genes but not of either gene individually produces limb defects (28). Similarly, deletions in SHFM1 patients identified so far invariably contain the *DLX5* and *DLX6* genes as well as the *DSS1* gene (29,30). Surprisingly, we identified a novel 880kb chromosomal deletion at 7q21.3 in a SHFM1 patient comprising *DSS1*, *SLC25A13* and part of *DYNC1I1* but leaving the *DLX5* and *DLX6* genes intact (Fig. 6A). Compared with the previously reported minimal chromosomal dele-

tion (29,30), the only full-length gene in the overlap region is *DSS1* which has been shown to be expressed in the mesenchyme of late developing limbs, craniofacial primordium and skin but not in ectoderm (30). Furthermore, there is no evidence so far that *DSS1* has a role in early stages of limb development. Therefore, we hypothesized that the p63 binding sites in the identified overlapping region are responsible for the limb defects in the SHFM1 patient. To test this hypothesis, we searched for p63 binding sites in this region and identified that binding site SHFM1-BS1 (Fig. 6A) was highly responsive to p63 in transient transfection assays. Transactivation activity was completely abolished by *p63* EEC mutations and by mutations in a p63 binding motif in the binding site (Fig. 6B). Moreover, we tested the genomic region containing this binding site in transgenic reporter assays in zebrafish and mice for their ability to control gene expression as regulatory elements. In both organisms, the reporter genes under the control of the chromosomal regions containing SHFM1-BS1 showed the same expression pattern in the apical ectodermal ridge (AER), ear and forebrain (Fig. 6C). Reporter expression in AER that directs limb (or fin) growth and patterning correlates perfectly with the expression of p63, DLX5 and DLX6 during embryonic limb development (11). Taken together, our data clearly demonstrated that p63 binding sites identified in HKCs can function as regulatory elements to control gene expression in embryonic limb development and suggest that disruption of proper gene regulation controlled by p63 binding sites leads to developmental defects.

Discussion

In the current study, we investigated the *p63* gene network in relation to the *p63* phenotype network to open new possibilities for elucidation of p63-related diseases. We took two genome-wide approaches to obtain DNA-binding and expression profiles in a physiologically relevant cell system to find target genes and regulatory elements of p63. Our list of target genes not only expands the repertoire of p63 target genes, but more importantly, it reveals novel p63 targets that are directly relevant to the disease mechanism. The association of many target genes with diseases that share similar features to p63-associated syndromes suggests that abnormal regulation of these target genes is responsible for the phenotypic defects in p63 patients. Furthermore, we showed that the binding sites identified in adult keratinocytes can regulate gene expression as regulatory elements during embryonic development, and that loss of these regulatory elements can lead to abnormalities in developmental disorders.

Although there is a clear overlap of binding sites in our ChIP-seq and the previous ChIP-on-chip study reported by Yang et al. (14), our data from HKCs appears to represent functional p63 binding sites more accurately. We have demonstrated that the large majority of the identified binding sites (more than 10,000 out of 11,425) contain a p63 motif which is very similar to that defined in the ChIP-on-chip analysis (14). This result indicates that p63 is directly bound to most of the binding sites in our study and not merely through association with protein complexes. Furthermore, we have observed that conservation of the binding sites (Fig. 3) and similarity of disease features associated with genes mapped by these binding sites (Fig. 5) are significantly higher than random expectation and they are only slightly lower than that from differentially regulated genes. Together, these data indicate that most binding sites obtained in this study are biologically functional.

In contrast to the large number of DNA-binding sites, our expression analysis detected only 328 genes differentially regulated in EEC patients (Fig. 2). There are several non-exclusive possibilities

to explain this difference. Firstly, the number of differentially regulated genes is small because only a subgroup of p63 target genes display commonly altered expression in analyzed EEC patients. In support of this hypothesis, GO annotation analysis of the differentially regulated genes in our study gave very specific functions related to ectodermal development (Supplementary Table 9). This is in contrast to general functional terms present in previous expression studies with RNAi knock-down of *p63* which might affect all p63 target genes (12,14-16). Secondly, mapping binding sites to genes is based on the distance between binding sites and genes, which is a commonly used method in current studies (14). However, as recent genome studies indicated, the regulatory elements such as the ones identified by p63 binding can function over long distance, and sometimes even over regions covering other genes (19). Some genes may also be regulated by multiple regulatory elements. Lastly, as our analyses (motif search, GO annotation, conservation and disease feature similarities) indicate that the majority of the identified p63 binding sites are functional, these binding sites may represent regulatory elements that function in other tissues than adult skin keratinocytes. In agreement with this hypothesis, our reporter analysis in animal models showed that binding sites identified in adult keratinocytes control gene expression in AER, ear and forebrain during embryonic development (Fig. 6), which suggests that p63 binding is partially tissue-independent. Consistently, our data on p53 DNA-binding and expression analyses showed that combining differentially expressed genes from several cell types increases the overlap with genes mapped by DNA-binding profiling (ML, unpublished results/ manuscript in preparation). Recently, tissue-specific binding sites have been reported for p300 (31), probably because DNA-binding of p300 represents activated transcription. Based on our data, we propose that DNA-binding of sequence-specific transcription factors such as p63 (and p53) is not sufficient for tissue-specific gene activation. DNA-binding and gene activation might be two separate events and p63 might bind to some target sites without activating the corresponding genes. This hypothesis is also supported by our observation that we have not found a clear difference in p63 binding motifs in up- versus down-regulated genes (Supplementary Fig. 2 and Fig. 6). Other transcription factors interacting with p63 may be involved in 'decision making' in a tissue- and developmental stage-specific pattern. We have not found over-represented motifs from other transcription factors in p63 binding sites, suggesting that other transcription factors do not bind to the same binding sites as p63. However, co-factors can also play a role in transactivation via protein-protein interactions.

Our functional analysis of the p63 binding site, SHFM1-BS1, has shown that this regulatory element can control expression of *DLX5/6* genes during embryonic limb development and loss of this control leads to limb defects in an SHFM1 patient (Fig. 6). Identification of this single regulatory element for both *DLX5* and *DLX6* is consistent with limb defects in mice being observed only when both genes are deleted (30). Interestingly, we also observed specific expression in the ear controlled by SHFM1-BS1 (Fig. 6). As deafness is also a feature observed in SHFM1 patients who have a deletion covering SHFM1-BS1 (32), our data are consistent with SHFM1-BS1 being important for inner ear development. These observations establish for the first time that disruption of a specific long-range regulatory element is the disease mechanism in SHFM1. This disease mechanism is novel and represents one of only three disease mechanisms responsible for SHFM known to date. Previously, SHFM3 was shown to be associated with partial *Dactylin* duplications (27) and SHFM4 to *p63* sequence mutations (4). The mechanisms for SHFM2 (33) and SHFM5 (34) remain unknown.

Another example of such regulatory elements identified by p63 binding sites is a p63 binding site upstream of *IRF6* that serves as an enhancer element to regulate expression of *IRF6* (manuscript

under review) (35). One SNP in this binding site has been associated with increased risk of cleft lip (35). Additionally, we found that more than 100 genes have been shown to be involved in clefting phenotypes in the mouse models whereas no mutations in these genes have been found in human diseases (Supplementary data). As many of these genes contain p63 binding sites, these sites can be used to screen for mutations or microdeletions that give rise to the clefting phenotype. Therefore, we propose that p63 binding sites can also be used as regulatory elements to study etiology of p63-related developmental disorders.

In summary, we have initiated elucidation of *p63* gene networks in relation to p63 disease phenotype networks. Our study has provided a comprehensive resource of potentially relevant genes as well as regulatory elements in p63-related diseases. We have also shown loss of the regulatory element SHFM1-BS1 identified by a p63 binding site as the novel disease mechanism for SHFM1. Identified target genes and regulatory elements of p63 can therefore be used as tools to screen for mutations and micro-deletions for unresolved p63-related diseases in both fundamental studies of disease mechanisms and clinical diagnostics.

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Author contributions

T.R., E.K., S.v.H. and H.Z. performed experiments/analyses and wrote the manuscript, H.Z. and H.v.B. designed experiments and wrote the manuscript, L.P. and E.B. performed expression validation and transient transfection experiments, L.S., M.L. and H.S. performed ChIP-seq analysis, C.G. and J.V. performed expression array analysis, A.H. performed CGH array analysis, B.H., H.B., T.R. and E.O. provided patients, S.T. and J.S. contributed to establish the primary keratinocyte culture, M.O., B.D. and M.H. performed data analysis on disease databases and evolutionary conservation, J.T., M.E.A., E.C-M., M.M. and J.L.G-S performed zebrafish and mice transgenic assays.

Methods

Human primary keratinocyte culture

Skin biopsies from the trunk were taken from EEC patients and healthy volunteers to set up the primary keratinocyte culture (36). Keratinocyte cultures in Keratinocyte Growth medium (KGM) was previously described (37).

RNA extraction and cDNA synthesis

RNA isolation and cDNA synthesis was performed as described (37). Details can be found in supplementary information.

Affymetrix expression array and data analyses

Quality of the total RNA was tested by using the Agilent 2100 Bioanalyzer (Agilent Technologies). Two microgram of total RNA from 48-hour differentiated keratinocytes was used for hybridizations on the Gene Chip Human Exon 1.0 ST Array (Affymetrix) according to the manufacturer's protocol (Gene Chip® Whole Transcript (WT) Sense Target Labeling Assay Manual). Partek® (Partek® Genomic Suite software, version 6.4 Copyright © 2008 Partek Inc., St. Louis, MO, USA) was used for extracting core exon expression and core transcript summaries were calculated using the mean intensities of the corresponding probe sets. The ANOVA model was applied on the log2 intensities and *P* values were generated for expression differences for the three pair wise comparisons of each of the individual patients versus the controls. Detailed data analysis can be found in supplementary information.

ChIP and ChIP-seq

ChIP experiments with HKCs were performed using a modified protocol as described (23) (details in supplementary information). ChIP-seq analysis was performed on a Solexa sequencing machine (Illumina) as described previously (38). Peak recognition was performed using MACS (20) with default settings and a *P* value threshold of 1E-9, giving 18,133 peaks and 14,963 peaks in ChIP-seq tracks of wt1 and wt2, respectively. Peaks were mapped to RefSeq genes, downloaded from the UCSC Genome Browser, to determine genomic location. The ChIP-seq data and associated peaks are deposited in NCBI's Gene Expression Omnibus (39) and are accessible through GEO Series accession number GSEXXXXX (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSEXXXXX).

Motif search

To determine the p63 motif, three motif prediction tools were run on 2285 (20%) randomly selected 200-bp peak sequences (centered at the peak summit as reported by MACS): MotifSampler (40), Weeder (41) and MDmodule (42). The significance of the predicted motifs was determined by scanning the remaining 80% of the peak sequences. *P* values were calculated using the hypergeometric distribution with the Benjamin-Hochberg multiple testing correction. All motifs with a *P* value < 0.001 and an absolute enrichment of at least > 1.5-fold compared to background were determined as significant. Details of motif search can be found in supplementary information. We calculated the ROC AUC for all significant motifs and chose the best performing motif based on the ROC AUC (Supplementary Table 8). The PWM of this motif was used with the p53scan algorithm and an optimal threshold, determined by the maximum f-measure as described previously (23). The p63scan algorithm can be dowloaded from http://www.ncmls.nl/bioinfo/p63scan/.

Quantitative PCR

Quantitative PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu). qPCR reactions were performed in the 7500 Fast Real Time PCR System apparatus (Applied Biosystems) by using iQ SYBRâ Green Supermix (BioRad) according to the manufacturer's protocol. Analysis of qPCR data

and qPCR primers for cDNA and ChIP analyses are described in supplementary information.

Analysis of potential target genes associated with phenotypic defects using human and mouse disease bases

The mapping of genes to diseases was taken from the Online Mendelian Inheritance in Man (OMIM) disease database (43). To identify overrepresented features, the OMIM free-text disease descriptions were first converted into lists of features using text mining (44). Further information can be found in supplementary information. Human diseases associated with orofacial clefting were taken from the Pictures Of Standard Syndromes and Undiagnosed Malformations (POSSUM) database (45) (POSSUM (c) Murdoch Children's Research Institute, Parkville, Victoria, Australia), and mapped to genes through their OMIM IDs. Mouse clefting-associated phenotypes and associated genes were taken from the Jackson Laboratory's Mouse Genome Database (http://www.informatics.jax.org/) (46).

Evolutionary conservation of binding sites

To assess the evolutionary conservation of the 11,425 sites bound by p63, the phastCons (47) conservation track was downloaded from the UCSC Genome Browser. Conservation based on 44 vertebrate genomes was chosen because the p63 gene has 1-1 orthologs throughout the vertebrates. The conservation for a region was calculated as the average conservation of each nucleotide therein.

Mapping deletion in a SHFM patient using ultra-high Comparative Genomic Hybridization

To detect chromosome 7 aberrations, high resolution NimbleGen HG18 chromosome 7 specific 385K arrays were used (B3738001-00-01; Roche NimbleGen Systems, Madison, Wisconsin, USA). The 385K average probe distance was 365bp. DNA labeling, array hybridization, post-hybridization washes and scanning were performed according to the manufacturer's instructions (Roche NimbleGen). The acquired images were analyzed using NimbleScan V2.4 extraction software (Roche NimbleGen). For each spot on the array, the log2 Cy3 (patient)/Cy5 (reference pool) ratio was calculated using the segMNT algorithm, which also applied an automatic segment detection. A 50x averaging window was generated, resulting in 20kb segments for this array. Breakpoints were determined with SignalMap V1.9 software (Roche NimbleGen) and 20kb averaged log2 ratios were visualized in the UCSC genome browser.

Constructs and transactivation assays

The genomic regions of p63 binding site peaks were cloned into a modified SmaI site in pGL3-Enhancer Vector with a firefly luciferase reporter gene followed by a SV40 enhancer. Wild-type and mutant p63 are cloned in a pcDNA backbone construct. Detailed information for the constructs is described in supplementary information. Transfection and luciferase assays were described previously (37).

Functional reporter analyses in zebrafish and mice

Human genomic fragments containing the SHFM1-BS1 p63 binding site were cloned into the ZED vector and transgenic zebrafish were generated as reported (48). Three or more independent stable transgenic lines were generated for each construct. Experimental details can be found in supplementary information. For the generation of transgenic mice, the genomic fragments were transferred into a vector containing the human minimal beta-globin promoter, lacZ and a SV40 polyadenylation signal. Constructs were linearized and the vector backbone removed prior to microinjection into the pronucleus of one-cell mouse embryos. F0 embryos of 11.5-13 dpc stages were harvested and stained for lacZ activity.

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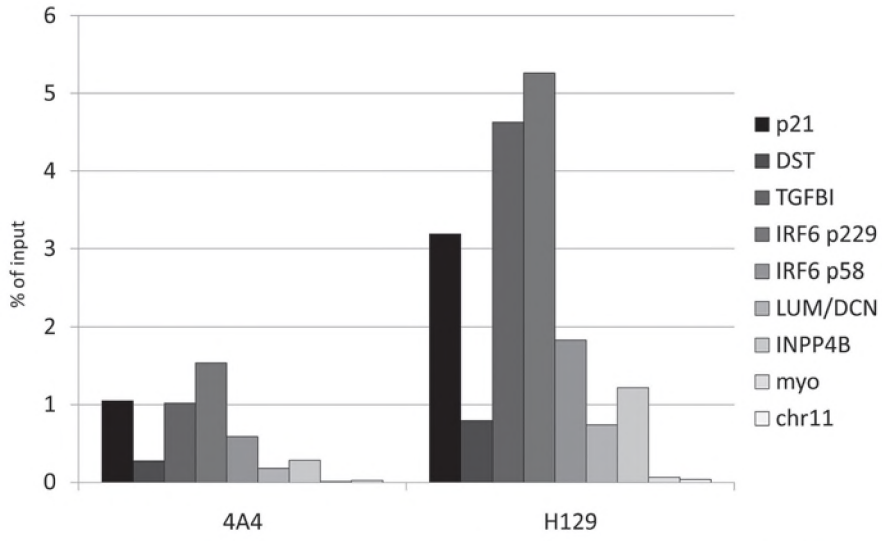
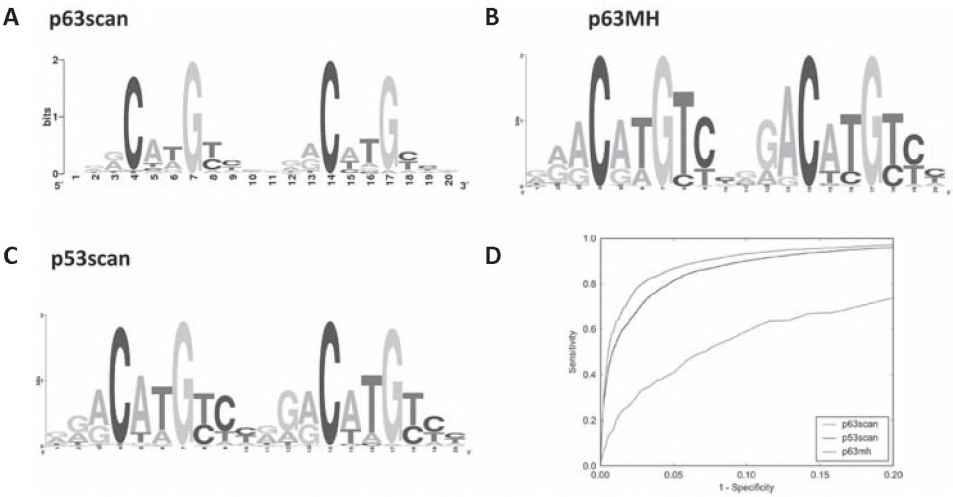
Supplementary information

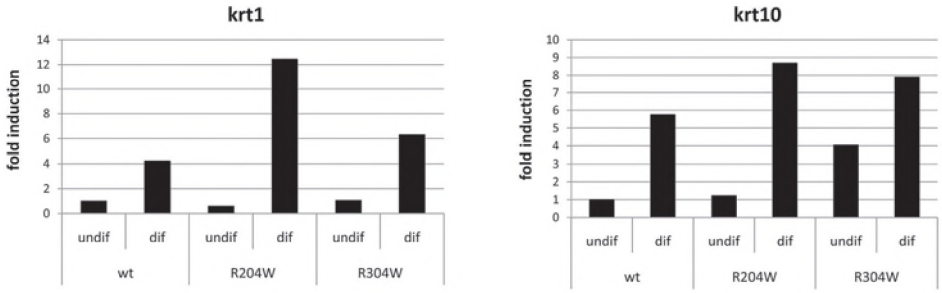
Clinical information. Patient 1 is a 29-year old woman who has dry depigmented skin and white hair. She has dystrophic nails and absence of enamel in her teeth. She is anhydrotic and has dacrocystitis secondary to nasolacrimal obstruction. She also has finger syndactyly and cleft lip and palate. She has a small dysplastic ears and hearing impairment, double sided hydronephrosis and narrowed urethra. She has frequent urinary tract infections. Mammary or nipple abnormalities have not been observed. This patient has been reported to carry a heterozygous p.R204W mutation in the DNA binding domain of the *p63* gene (1). Patient 2 is a 51-year old man, who has a very dry and scaling skin in the nose, back of the hands and back. He has a history of a malignant spinocellular epithelioma in his left hand that has been exposed to the sun because of his daily work outside. He has dental Prosthesis, his nails are hypoplastic and he has no hair. He has corrected dacryostenosis and cleft lip and palate. He has syndactyly and ectrodactyly in his hands and feet. He also has several genito-urinary abnormalities with a double pelvo-uretieric system on the right side, narrowed bladder neck, prostate hypertrophy and hypospadias. He has normal sweating and normal nipples. This patient has been reported to carry a heterozygous p.R279H mutation in the DNA binding domain of the *p63* gene (2). Patient 3 is a 59-year old man who has a dry, but normally sweating skin. His hair is coarse and he has dental prostheses. He has a corrected dycryostenosis on the left side and absence of Meibomian glands in the eye. He has ectrodactyly and syndactyly in one hand and foot. And he also has left sided cleft lip and palate. He suffers from hearing impairment because of narrowed ear canal and has a history of chronic otitis media and a cholesteatoma. He also has several genito-urinary abnormalities: hypospadias, prostate hypoplasia, chronic interstitial nephritis and a broadened urethra. This patient has been reported to carry a heterozygous p.R304W mutation in the DNA binding domain of the *p63* gene (3). Patient 4 has typical phenotype of Split hand/foot malformation (SHFM) and his DNA was used in CGH deletion analysis.



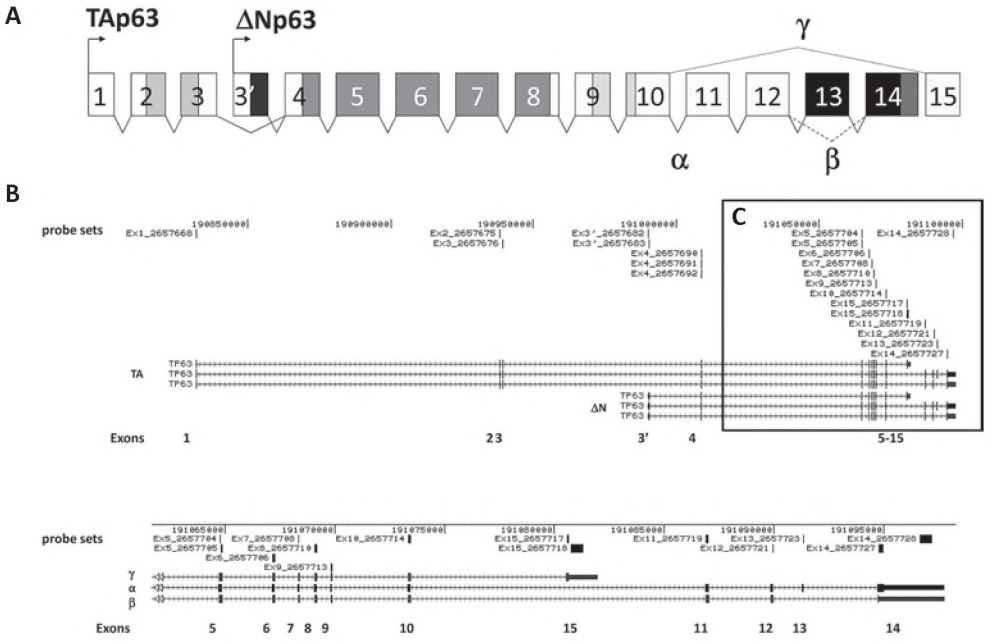
Supplementary figure 1. ChIP-qPCR analysis of p63 binding in human primary keratinocytes using two different p63 antibodies 4A4 (pan-p63) and H129 α-specific). Specific binding of p63 to the tested binding sites was observed, including to binding sites at $p21^{WAF/CIP19}$ and DST which served as positive controls, but not to the negative controls myoglobin exon 2 (myo) and a no-gene region (chr11).



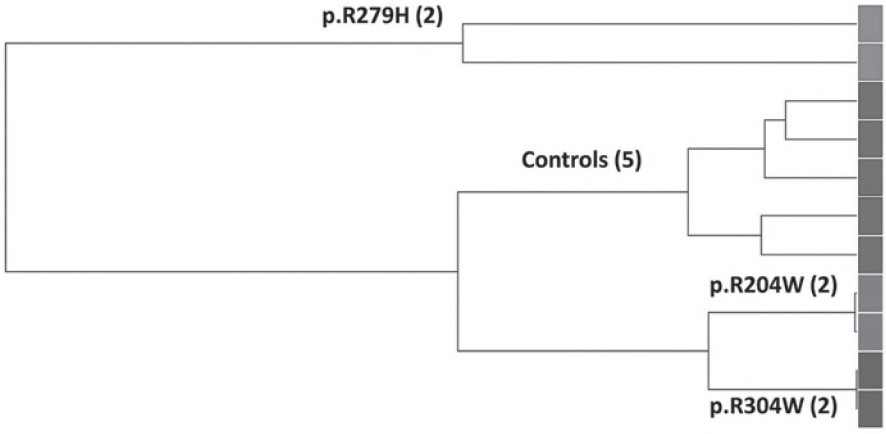
Supplementary figure 2. Comparison of the p63 motif identified in this work with previously reported motifs. **A.** The p63 motif was identified by de novo motif analysis where 20% of the binding sites were used to predict motifs, and the resulting motifs were scored for significance using the remaining 80% of the binding sites compared to a set of background sequences. Based on a previously developed p53scan algorithm (4) with this newly identified p63 Positional Weight Matrix (PWM), p63scan was developed. **B-C.** The motifs from p63MH (5) and p53scan (4), respectively. **D.** The performance of p63scan using the de novo identified motif shows far superior sensitivity compared to previously reported p63MH without compromising specificity. Compared to p53scan, which was deduced from p53 binding sites, p63scan performs also slightly better on p63 binding sites. The 80% of the binding site sequences not used for motif prediction, as well as a set of background sequences were scanned using the different algorithms. For each individual threshold the fraction of binding sites with a motif (sensitivity) as well as the fraction of background sequences with a motif (1 – specificity) were calculated. For a color figure see Appendix 2.



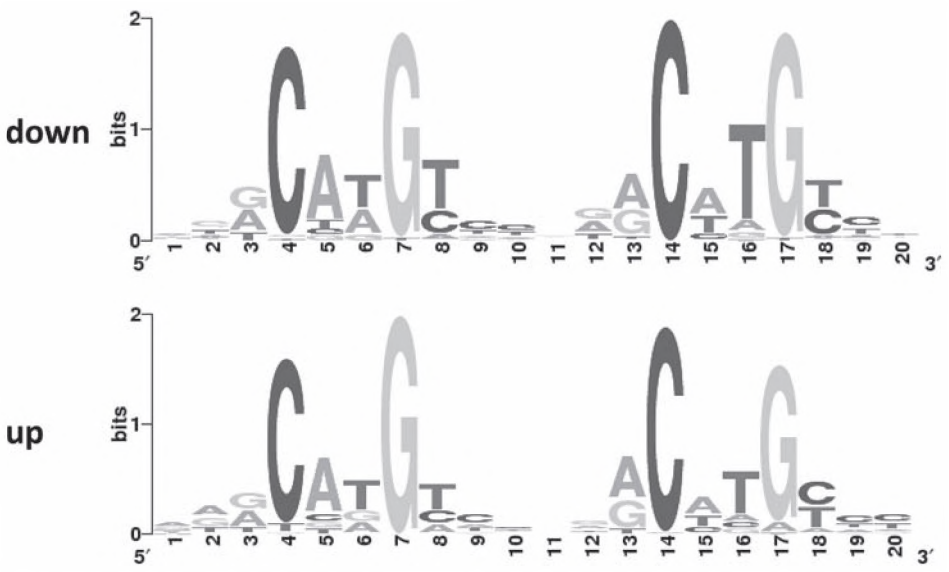
Supplementary figure 3. Expression of early differentiation markers and in differentiated primary keratinocytes. At 48-hour differentiation, the early differentiation markers, Keratin-1 and Keratin-10, were expressed similar in human primary keratinocytes established with a control individual and patients with p.R204W and p.R304W *p63* mutations.



Supplementary figure 4. Expression analysis of individual exons of the *p63* gene. A. Diagram of the genomic structure of the *p63* gene indicates the most commonly mentioned six isoforms of p63: two alternative promoters give rise to N-terminal TA- and ΔN- isoforms; three different splicing routes give rise to α, β, and γ C-terminal isoforms. **B.** A screenshot of genomic structure of the *p63* gene shows the core probe sets used to determine exon expression with their corresponding exons. **C.** The core probe sets and their corresponding exons are shown in a zoomed-in screenshot at the 3' end of the *p63* gene. For a color figure see Appendix 2.



Supplementary figure 5. Hierarchical clustering of the expression array samples. Hierarchical clustering analysis showed that expression pattern of five normal control samples were very similar to each other even though they were established from 5 different individuals. The replicates of each mutant clustered together and the expression pattern of p.R204W and p.R304W mutant cell lines were more similar to each other compared to the mutant p.R279H.



Supplementary figure 6. Motif analysis of binding sites near up- or down regulated genes. The de novo motif search on binding sites of up- or down-regulated genes revealed no major difference in p63 motifs. For a color figure see Appendix 2.

Supplementary Table 1. Validation by ChIP-qPCR of detected binding sites in ChIP-seq analysis.

Chromosomal location	Potential target gene	ChIP-seq		ChIP-qPCR[a]	
		Peak height	Peak size	wt1	wt2
chr7:92496280-92496740	*CDK6*	286	460	6/6	4/4
chr1:208055902-208056327	*IRF6*	229	426	6/6	4/4
chr6:123135710-123135865	*FABP7*	108	156	4/4	4/4
chr20:29662160-29662520	*ID1*	93	271	4/4	4/4
chr10:85928813-85929380	*PCDH21*	87	568	4/4	
chr11:74952517-74952815	*map6*	78	299	4/4	
chr12:97562240-97562680	*IKIP*	53	441	4/4	
chr4:143545950-143546350	*INPP4b*	49	400	6/6	4/4
chr11:124889250-124889900	*FEZ1*	42	330	4/4	
chr1:10057464-10057816	*UBE4B*	24	353	2/6	
chr4:143942600-143942970	*INPP4b*	22	371	4/8	
chr7:107365280-107365520	*LAMB1*	17	341	2/2	4/4
chr6:7586275-7586580	*BMP6*	16	306	4/4	4/4
chr6:7827300-7827500	*BMP6*	15	341	4/4	
chr3:187276440-187276780	*ETV5*	15	201	4/6	
chr11:75112380-75112720	*MAP6*	12	330	3/6	
chr7:107444700-107444900	*LAMB1*	11	201	6/6	

[a] Independent ChIP-qPCR experiments were performed multiple times to validate detected binding sites. As the first step, 17 binding sites with the peak heights ranging from 286 to 11 were chosen to be validated in one of the primary cell lines wt1, and binding of p63 was unambiguously confirmed at 16 out of 17 tested binding sites. Seven out of these 16 confirmed peaks were then again tested in the other cell line wt2 in which p63 binding was also confirmed. The numbers below wt1 and wt2 indicate the number of experiments in which p63 binding was detected out of the total number of independent experiments. For example, 6/6 means that p63 binding was shown in all 6 independent ChIP-qPCR experiments. Whether binding detected or not is based on the fold enrichment of 3 over an internal negative control regions to which p63 does not bind, myoglobin exon 2 region or a no-gene region on chromosome 11.

Supplementary Table 2. GO annotationsa of genes mapped by binding sites in ChIP-seq analysis.

GO No.	Term	No. of genes	P Value[b]
GO:0032502	Developmental process	1356	4.95E-39
GO:0048856	Anatomical structure development	900	2.10E-25
GO:0007275	Multicellular organismal development	958	5.91E-23
GO:0030154	Cell differentiation	774	6.01E-23
GO:0048869	Cellular developmental process	774	6.01E-23
GO:0065007	Biological regulation	1939	3.12E-22
GO:0048519	Negative regulation of biological process	527	1.84E-21
GO:0048731	System development	735	2.03E-20
GO:0048523	Negative regulation of cellular process	501	1.49E-19
GO:0050789	Regulation of biological process	1754	1.70E-18
GO:0016043	Cellular component organization and biogenesis	1062	3.97E-18
GO:0007242	Intracellular signaling cascade	626	1.33E-17
GO:0022610	Biological adhesion	356	4.57E-17
GO:0007155	Cell adhesion	356	4.57E-17
GO:0009987	Cellular process	4314	1.16E-15
GO:0048468	Cell development	522	1.62E-14
GO:0050794	Regulation of cellular process	1612	4.97E-14
GO:0048513	Organ development	530	1.86E-13
GO:0048518	Positive regulation of biological process	466	1.99E-13
GO:0016265	Death	365	2.81E-13
GO:0008219	Cell death	365	2.81E-13
GO:0043687	Post-translational protein modification	615	2.92E-13
GO:0043412	Biopolymer modification	740	3.34E-13
GO:0006464	Protein modification process	713	5.77E-13
GO:0012501	Programmed cell death	345	2.32E-12
GO:0006915	Apoptosis	342	2.87E-12
GO:0051179	Localization	1174	3.88E-12
GO:0048522	Positive regulation of cellular process	417	1.29E-11
GO:0009653	Anatomical structure morphogenesis	466	1.74E-11
GO:0007167	Enzyme linked receptor protein signaling pathway	144	9.99E-11

[a] Gene Ontology (GO) annotations was performed using DAVID Bioinformatics Resources (NIH) on 10,852 genes which 11,425 detected binding sites were mapped to.

[b] P value was obtained using default setting in DAVID without multiple testing. GO terms which have P values below 10^{-10} are shown in the table.

Supplementary Table 3. Significant motifs predicted using a de novo motif prediction pipeline (MotifSampler, Weeder and MDmodule).

Motif Consensus	Program	Motif family	P value (genomic)	Enrichment (genomic)	P value (random)	Enrichment (random)	ROC AUC	Max f-measure
nrCAwGyynnnrCwTGyn	MotifSampler	p63	0.00E+000	88.7	0.00E+000	39	0.97	0.919
nnrCAwGynnnnrCwTGynn	MotifSampler	p63	0.00E+000	96.6	0.00E+000	50.4	0.97	0.916
rCAwGynnnnrCwTGy	MotifSampler	p63	0.00E+000	61.3	0.00E+000	25.1	0.97	0.911
CAwGynnnnrCwTG	MotifSampler	p63	0.00E+000	24.7	0.00E+000	11.9	0.95	0.882
nACATGTnnnAACwTGyn	MDmodule	p63	0.00E+000	129.6	0.00E+000	91	0.95	0.884
ACAwGTynnrACwT	MDmodule	p63	0.00E+000	16.5	0.00E+000	10.5	0.89	0.82
nGynnrnrCwTG	MotifSampler	p63	0.00E+000	2.8	0.00E+000	2.2	0.89	0.824
rnrCATGyyn	MotifSampler	p63	0.00E+000	2.6	0.00E+000	1.6	0.8	0.754
TGTCCAGACATG	Weeder	p63	0.00E+000	31.3	0.00E+000	14.4	0.8	0.743
CAwGTynnrACw	MDmodule	p63	0.00E+000	9.5	0.00E+000	6.2	0.79	0.735
GACATGCC	Weeder	p63	0.00E+000	4.5	0.00E+000	2.8	0.78	0.733
GGCATGTC	Weeder	p63	0.00E+000	4.4	0.00E+000	2.7	0.78	0.732
GACATGTC	Weeder	p63	0.00E+000	5.6	0.00E+000	3.3	0.77	0.738
GGCATGTT	Weeder	p63	0.00E+000	4.5	0.00E+000	2.8	0.76	0.741
nnAACATGCC	Weeder	p63	0.00E+000	5.4	0.00E+000	4.9	0.76	0.73
TAGGCATGTC	Weeder	p63	0.00E+000	5.4	5.04E-297	4.6	0.75	0.72
GACATGTCCA	Weeder	p63	8.44E-253	6.2	1.56E-133	3.6	0.74	0.714
TnGACATGTC	Weeder	p63	0.00E+000	6.8	1.36E-184	4.2	0.74	0.721
ACATGC	Weeder	p63	0.00E+000	3.1	0.00E+000	2.4	0.74	0.735
GCATGT	Weeder	p63	0.00E+000	3.1	0.00E+000	2.4	0.74	0.735
AACATGTC	Weeder	p63	0.00E+000	4.3	0.00E+000	3	0.73	0.708
CAGACATGTC	Weeder	p63	1.47E-293	5.8	1.31E-184	3.9	0.73	0.708
ACATGT	Weeder	p63	0.00E+000	3.1	0.00E+000	2.4	0.72	0.703
GACATG	Weeder	p63	0.00E+000	1.8	0.00E+000	1.7	0.7	0.709
CATGTC	Weeder	p63	0.00E+000	1.8	0.00E+000	1.7	0.7	0.714
AACAwGTy	MDmodule	p63	0.00E+000	3	0.00E+000	2.6	0.65	0.673
wGTTsAnACw	MDmodule	p63	1.40E-226	2	1.41E-221	1.9	0.6	0.67
CACACACACACACACACA	MDmodule	CA-repeat	2.46E-009	1.6	0.00E+000	490	0.6	0.669
CTTGTynTTwCT	MDmodule	-	2.47E-057	1.8	1.93E-154	2.8	0.57	0.669
nAmTGAGTnAnT	MDmodule	Fos/AP1	1.21E-068	1.7	1.30E-069	1.8	0.57	N/A
AnTnACwAGTTCATAC	MDmodule	-	4.47E-018	7	4.14E-023	9	0.55	0.668

Supplementary Table 4. General EEC phenotype and the clinical picture of the three EEC patients used in this study.

	General EEC phenotype	Patient 1 p.R204W	Patient 2 p.R279H	Patient 3 p.R304W
ED				
Skin	34%	+	+	+
Hair	66%	+	+?	+
Teeth	53%	+	+	+
Nails	52%	+	+	-/+
Mammary glands	14%	-?	-	-
Sweat gland	11%	+	-?	-?
Lacrimal ducts	57%	+	+	+
Corneal dystrophy	?	?	+	+
Limbs				
Ectrodactyly	68%	-	+	+
Syndactyly	43%	+	+	+
Orofacial clefting				
Cleft lip	39%	+	+	+
Cleft palate	40%	+	+	+
Others				
Hearing impairment	7%	+	-	+
Genito-urinary problems	15%	+	+	+

Supplementary Table 5. Gene with significant expression changes ($P < 0.05$) in EEC patient keratinocytes *(Appendix 1)*

Supplementary Table 6. Genes with consistent significant expression changes more than 1.5-fold *(Available on request)*

Supplementary Table 7. Validation by qPCR of down-regulated genes obtained in expression array.

Genes	Mutations	Array data	Validation by qPCR	
		Fold change	*Fold change*	*Std*
GPX2	p.R279H	28.0	37.7	±6.1
	p.R204W	25.5	35.3	±8.3
	p.R304W	12.9	8.2	±0.4
ADH7	p.R279H	16.4	21.2	±2.8
	p.R204W	15.7	30.7	±6.7
	p.R304W	13.9	33.4	±9.2
GBP6	p.R279H	15.1	9.6	±1.8
	p.R204W	9.1	7.6	±3.0
	p.R304W	8.2	2.6	±0.6
CTSK	p.R279H	10.0	9.5	±3.3
	p.R204W	9.9	9.3	±0.1
	p.R304W	5.4	5.0	±0.7
ETV4	p.R279H	3.4	2.2	±1.2
	p.R204W	3.0	2.1	±0.1
	p.R304W	3.4	2.9	±0.8
FZD7	p.R279H	3.3	9.8	±2.9
	p.R204W	2.0	3.0	±0.1
	p.R304W	1.6	3.3	±0.0
PORC	p.R279H	2.8	3.2	±0.9
	p.R204W	2.2	2.7	±0.0
	p.R304W	2.5	5.4	0.2
CLDN7	p.R279H	1.9	3.3	±1.0
	p.R204W	1.7	1.2	±0.0
	p.R304W	1.7	1.7	±0.0
IRF6	p.R279H	2.3	2.5	±0.4
	p.R204W	1.6	1.1	±0.2
	p.R304W	1.7	1.1	±0.3
CTNNB	p.R279H	1.9	1.7	±0.4
	p.R204W	1.6	2.5	±0.6
	p.R304W	1.4	3.5	±0.0

Supplementary Table 8. Validation by qPCR of up-regulated genes obtained in expression array.

Genes	Mutations	Array data	Validation by qPCR	
		Fold change	*Fold change*	*Std*
FABP7	p.R279H	651.1	87.1	±10.2
	p.R204W	76.5	82.7	±2.3
	p.R304W	15.4	90.6	±1.9
METTL7A	p.R279H	5.7	2.4	±0.6
	p.R204W	5.2	4.5	±0.1
	p.R304W	7.6	3.7	±0.2
LAMB1	p.R279H	2.8	2.0	±0.6
	p.R204W	3.0	2.7	±0.0
	p.R304W	3.0	3.0	±0.0
ID2	p.R279H	2.9	2.0	±0.6
	p.R204W	2.5	1.2	±0.0
	p.R304W	3.0	2.6	±0.2
ID3	p.R279H	2.4	1.1	±0.4
	p.R204W	1.9	2.0	±0.0
	p.R304W	2.1	2.0	±0.2
TGFBI	p.R279H	1.9	1.9	±0.3
	p.R204W	1.8	2.7	±0.5
	p.R304W	1.8	1.9	±0.5

Supplementary Table 9. GO annotationsa of differentially regulated genes in EEC keratinocytes.

GO No.	GO term	No. of genes	P Value[b]
GO:0007398	Ectoderm development	11	1.69E-04
GO:0002009	Morphogenesis of an epithelium	8	2.99E-04
GO:0048513	Organ development	35	0.001116
GO:0008544	Epidermis development	9	0.002003
GO:0009888	Tissue development	14	0.002224
GO:0048856	Anatomical structure development	50	0.002496
GO:0007275	Multicellular organismal development	53	0.003247
GO:0048731	System development	42	0.00393
GO:0032502	Developmental process	68	0.004811

[a] Gene Ontology (GO) annotations was performed using DAVID Bioinformatics Resources (NIH) on 328 significantly changed genes obtained from expression array analysis of five normal controls and three EEC patient keratinocyte cell lines.

[b] P value was obtained using default setting in DAVID without multiple testing. GO terms which have P values below 0.005 are shown in the table.

Supplementary Table 10. Differentially regulated genes with binding sites nearby

(Available on request)

Supplementary Table 11. GO annotationsa of potential direct target genes.

GO No.	GO term	No. of genes	*P* Value[b]
GO:0007398	Ectoderm development	8	7.46E-04
GO:0048513	Organ development	25	8.19E-04
GO:0048731	System development	30	0.001697
GO:0048856	Anatomical structure development	34	0.002583
GO:0008544	Epidermis development	7	0.002711
GO:0065007	Biological regulation	66	0.003765
GO:0000074	Regulation of progression through cell cycle	13	0.003961
GO:0051726	Regulation of cell cycle	13	0.004144
GO:0050789	Regulation of biological process	61	0.004171
GO:0048523	Negative regulation of cellular process	21	0.004463
GO:0009888	Tissue development	10	0.004693

[a] Gene Ontology (GO) annotations was performed using DAVID Bioinformatics Resources (NIH) on 194 genes which have changed expression in EEC keratinocytes and a p63 binding sites near by. [b] *P* value was obtained using default setting in DAVID without multiple testing. GO terms which have *P* values below 0.005 are shown in the table.

Supplementary Table 12. Association of diseases with genes identified in ChIP and expression analysis

(Available on request)

Supplementary Table 13. Genes are known to be involved in cleft lip/palate

(Available on request)

Supplementary Table 14. Primers for expression, ChIP analyses and for cloning

(Available on request)

Supplementary methods

RNA extraction and cDNA synthesis

HKCs at 48-hour of differentiation were used for the expression analysis. At this stage, differentiation markers were similarly expressed in normal and patient keratinocytes (Supplementary Fig. 3). Total RNA was transcribed into cDNA according to the manufacturer's protocol using reverse transcriptase PCR iScriptTM cDNA Synthesis kit (BioRad). The cDNA produced was purified by NucleoSpin Extract II kit (Macherey-Nagel, Bioke) according to the manufacturer's protocol.

Affymetrix expression array data analyses

The Affymetrix cel-files were first imported into Affymetrix Expression Console version 1.1 where control probes were extracted using the default RMA algorithm in order to perform quality analysis checks. The Area Under the Curve (AUC) of the Receiver Operator Characteristic was calculated using the positive and negative control probes. All arrays had AUC score above the empirically defined threshold of 0.85 indicating a good separation of the positive controls from the negative controls. Subsequently the cell-files were imported into Partek® (Partek® Genomic Suite software, version 6.4 Copyright © 2008 Partek Inc., St. Louis, MO, USA) where only core exons were extracted and normalized using the RMA algorithm with GC background correction. Core transcript summaries were calculated using the mean intensities of the corresponding probe sets. The correspondence of the replicate samples was confirmed using Principle Component Analysis (PCA) and Pearson correlation analysis. After grouping the samples by patients, an analysis of variance components indicated that the scan date of the samples had a relatively large influence on the expression profiles and this was thus used as an additional factor in the Anova model. Applying the Anova model on the log2 intensities we generated *P* values for expression differences for the three pair wise comparisons of each of the individual patients versus the controls. In each comparison we selected only genes with *P* values ≤ 0.05 and an expression difference of at least 1.5 fold. From the intersection of these three lists we then selected only the genes that showed the same direction of expression change in all three patients compared to the controls.

ChIP and ChIP-seq

Human primary keratinocytes under proliferating condition where p63 is expressed at the highest level were used for ChIP and ChIP-seq analysis. were crosslinked with 1% formaldehyde for 10 minutes and chromatin was collected as described (6). Chromatin was sonicated using a Bioruptor sonicator (Diagenode) for 2 times of 8 minutes at high power, 30s ON, 30s OFF. p63 antibodies 4A4 (Abcam) and H129 (Santa Cruz) were used in targeted ChIP experiments and 4A4 was used for ChIP-seq analysis. ChIP experiments were performed as previously described (4). ChIP-seq analysis was performed on a Solexa sequencing machine (Illumina) as described previously (7). All 32-bp sequence reads were uniquely mapped to the human genome NCBI build 36.1 (hg18) with zero or one mismatch using ELAND (Illumina), resulting in 3.2 and 6 million unique reads for the two analyzed samples, wt1 and wt2 respectively. Peak recognition was performed using algorithm of Model-based Analysis of ChIP-seq (8) with default settings and a *P* value threshold of 1e-9, giving 18,133 peaks and 14,963 peaks in ChIP-seq tracks of wt1 and wt2, respectively. Peaks were mapped to RefSeq genes, downloaded from the UCSC Genome Browser, to determine genomic location.

Motif search

To determine the p63 motif, three motif prediction tools were run on 2285 (20%) randomly selected 200-bp peak sequences (centered at the peak summit as reported by MACS (8)): MotifSampler (9), Weeder (10) and MDmodule (11). We used the 'large' analysis setting for Weeder. MDmodule and MotifSampler were each used to predict 10 motifs for each of widths between 6 and 20. The significance of the predicted motifs was determined by scanning the remaining 80% of the peak sequences and two different backgrounds: a set of random genomic sequences with a similar genomic distribution as the peak sequences and a set of random sequences generated according to a first order Markov model, match in the dinucleotide frequency of the peak sequences. *P* values were calculated using the hypergeometric distribution with the Benjamin-Hochberg multiple testing correction. All motifs with a *P* value < 0.001 and an absolute enrichment of at least > 1.5-fold compared to both backgrounds were determined as significant. We calculated the ROC AUC for all significant motifs and chose the best performing motif based on the ROC AUC (See Supplementary Table 8 for the results). The PWM of this motif was used with the p53scan algorithm and an optimal threshold, determined by the maximum f-measure as described previously (4). The p63scan algorithm can be downloaded from http://www.ncmls.nl/bioinfo/p53scan/.

Gene Ontology Annotations

Gene Ontology analysis on differentially expressed genes and genes mapped by the nearby binding sites was performed using DAVID Bioinformatics Resources (NIH) (http://david.abcc.ncifcrf.gov). *P* value was obtained using default setting in DAVID without multiple testing.

Quantitative PCR analysis

For qPCR of cDNA analysis, two or three exon-spanning primer sets for each validated gene were used (Supplementary Table 14). *Human acidic ribosomal protein (hARP)* and *Hypoxanthine phosphoribosyl-transferase* (*HPRT*) were used as a housekeeping gene to normalize the amount of cDNA. Differences in expression of each gene between wild-type and mutant samples (relative expression) were calculated by $2^{\Delta\Delta Ct}$ method (12,13). The relative expression was averaged using two or three primer sets. For qPCR of ChIP analysis, one primer set was used for each tested binding region (Supplementary Table 14) and ChIP efficiency of certain binding sites was calculated using percentage of ChIPped DNA against input chromatin.

Analysis of potential target genes associated with phenotypic defects using OMIM disease bases

The mapping of genes to diseases was taken from the Online Mendelian Inheritance in Man (OMIM) disease database (14,15), downloaded on December 19th, 2008. In order to identify over-represented features, the OMIM free-text disease descriptions were first converted into lists of features using text mining (16). Disease features were defined using the "Anatomy" (A) and "Pathological Conditions, Signs and Symptoms" (C23) categories of the 2008 version of the Medical Subject Headings hierarchical controlled vocabulary (http://www.nlm.nih.gov/mesh/meshhome.html), excluding the broad first-level terms. Feature overrepresentation in the target gene-associated diseases relative to the full OMIM database was determined using the hypergeometric enrichment test as implemented in the R statistical software package (http://www.r-project.org/).

Constructs for transactivation assays

The genomic regions of p63 binding site peaks were amplified by PCR with gateway cloning primers and cloned into a modified SmaI site in pGL3-Enhancer Vector which contains a firefly luciferase reporter gene followed by a SV40 enhancer (4). The cloning primers are described in Supplementary Table 14. Point mutations were introduced into p63-binding motifs of SHFM1-BS1 to generate mutant p63 binding sites, where the essential cytosine and guanine bases were mutated to adenosine. The ΔNp63α wild-type (Mm_pcDNA_ΔNp63α) expression plasmid has been described previously (17). Point mutations were introduced into this plasmid to generate p.R204W, p.R279H and p.R304W mutations.

Functional reporter analyses in zebrafish and mice

Human genomic fragments containing the SHFM1-BS1, SHFM1-BS2 and SHFM1-BS3 p63-bound regions were amplified with the primers: SHFM1-BS1F: 5'-CCACAGGGCCTTGTAAACG-3'; SHFM1-BS1R: 5'-GATAAAACTCTACCTTGTGGGAGGC-3'; SHFM1-BS2F: 5'-CCAGCCTAAAAATTCACAGTGC-3'; SHFM1-BS2R: 5'-GGTGGAAGCAGCCTGAGG-3'; SHFM1-BS3F: 5'-CGGTGACGGAGTGATACCC-3'; SHFM1-BS3R: 5'-GAGACTATCCCCCTTCAGGTAGG-3'. The PCR fragments, of 1780, 1682 and 573 pb, respectively, were subcloned in PCR8/GW/TOPO vector and then transferred, through recombination using Gateway technology, to the ZED destination vector for zebrafish transgenesis (18). This vector contains the Xenopus Cardiac actin promoter driving DsRed as a positive control for transgenesis. To generate the zebrafish transgenic embryos, we used Tol2 transposon/transposase method (19) with minor modifications. 1nl was injected in the cell of 1 cell stage embryos containing 50ng/ul of transposase mRNA, 40ng/ul of phenol/chloroform purified ZED constructs and 0.05% phenol red. Three or more independent stable transgenic lines were generated for each construct. For the generation of transgenic mice, the genomic fragments were transferred into a vector containing the human minimal beta-globin promoter, lacZ and a SV40 polyadenylation signal. Constructs were linearized and the vector backbone removed prior to microinjection into the pronucleus of one-cell mouse embryos. F0 embryos of 11.5-13 dpc stages were harvested and stained for lacZ activity.

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General discussion and future prospects

8.1 p63 isoform specificity in development and disease

During the past few years important new insight has been obtained into some general molecular and cellular functions of p63. However, understanding of the function of all different p63 isoforms and their putative reciprocal interactions has turned out to be a complex but necessary following step. Currently it is known that TA- and ΔN isoforms are regulated by different promoters, their expression pattern is different, and they can both activate and repress the transcription of target genes (1-6). ΔNp63 isoforms are prominently expressed in different epithelial (basal, squamous and myoepithelium) tissues e.g. in epidermal, oral, exocrine glands, thymus and urogenital tissues (5,7-9). Within the epithelia, ΔNp63 isoforms can be found in the basal cell layer (10). All but one study (11) show that ΔNp63α is the first p63 isoform that is expressed in the developing epidermis (1,11-13). This isoform is most likely important for maintenance of the stem cell identity and proliferation (1,11-13). Although TA isoforms have been detected in the epidermis as well, their role in epidermal development appears to be less important than that of the ΔNp63 isoforms as demonstrated by the lack of ectodermal defects in TA-deficient mice (1,14). We and others have demonstrated that AEC and Rapp Hodgkin syndrome mutations are clustered in the ΔNp63α isoforms (TA2, SAM and TI domains) (Chapters 5 and 6), which supports the important role of ΔNp63α in epithelia and ectodermal development.

In contrast to the ΔNp63α isoforms, specific expression and function of the TAp63 isoforms have not been linked to tissues that are generally affected in p63-associated syndromes. Accordingly, only one TA-specific mutation has been identified so far. This mutation was found in an isolated SHFM patient carrying a missense mutation in the TA-domain (15). Although TA isoforms have been detected in the developing epidermis in a few studies, they appear not to be involved in the maintenance process of the epidermal stem cells. However, TA isoforms may have a role in the terminal epidermal differentiation by counteracting the activities of ΔNp63α isoforms (1,13,14,16). TAp63 isoforms have been detected in heart, testis, kidney, brain and thymus, but the relevance for patients with a *p63* mutation has not been established (5,10,17,18). High expression of TAp63α isoforms has also been detected in primordial germ cells (oocytes), where they have a role in DNA damage response (8,14). More studies suggest a role for TA isoforms to be involved in pre-apoptotic pathways, e.g. during neural development (18-21). However, no association has been detected between p63-syndrome patients and neuronal defects, suggesting a less important role for the TAp63 in the p63-linked disease pathogenesis. Whether the apoptosis-induction of TAp63 isoforms may contribute to other developmental processes remains to be established by careful analysis of the TA-specific knockout.

Our published (Chapter 6 and 7) and unpublished studies on the epidermis of p63-linked patients confirm the expression pattern of ΔNp63α: ΔNp63α is clearly expressed in the basal and suprabasal cells, but absent in the upper epidermal layers. We were not able to detect TAp63 isoforms in skin biopsies, nor in the cultured human keratinocytes. However, we detected a new isoform ΔΔN63α that lacks the first 26 amino acids of the amino-terminal TA2 domain in cultured human keratinocytes (Chapter 6). The ΔΔNp63α variant is expressed in human control keratinocytes at low levels, whereas its expression is similar to the longer ΔNp63α in cells derived from AEC/RHS patients carrying amino-terminal truncating mutations. Possibly, this new isoform is a natural dominant-negative regulator of ΔNp63α, suggesting that maintaining the balance between ΔNp63α and ΔΔNp63α is crucial for the proper ectodermal development.

8.2 Clinical variability in p63-associated syndromes and disease mechanism

The production of clinically different phenotypes from mutations in the same gene, clinical heterogeneity, is the rule rather than the exception in medical genetics. The clinical variability is most pronounced when mutations affect different functional domains or when they have opposite functional consequences. For instance, loss-of-function mutations in the tyrosine kinase domain in the *Fibroblast growth factor 2 (FGFR2)* cause Lacrimo-auriculo-dento-digital syndrome (LADD, OMIM 149730) (22), whereas gain-of-function mutations in immunoglobulin domains in the same gene lead to syndromic forms of craniosynostosis (22-25). Similarly, mutations that are clustered in different p63 functional domains have probably different effects on the protein leading to different functional consequences and finally to different clinical phenotypes. Results from our own research and that of other groups have revealed that EEC and ADULT syndrome mutations cluster in the DNA binding domain and AEC/RHS mutations either in the TA2, SAM or TI domain. For other p63-related diseases a specific domain clustering has not yet clearly been established. This is probably because the number of patients in other p63-related disorders is small, or because they cluster in specific yet unrecognized functional sites in p63 protein.

Clinical variability has not only been described between the various p63-linked syndromes, but also among patients within one p63-linked disease. In EEC syndrome, for example more than 80 percent of the patients carrying the p.R304 mutation have orofacial clefting, but hardly any of the patients carrying the p.R227 have cleft lip/palate (CLP). Moreover, I have demonstrated that clinical variability is even apparent for phenotypes that are caused by the same mutation (Chapter 3). Analysis of the five EEC hot spot mutations revealed that all symptoms display variable expressivity within patients, which is not restricted to EEC syndrome but is seen for all p63-associated conditions. What are the molecular determinants of clinical variability? For the range of syndrome-specific mutations, it is possible that the various mutations exhibit overlapping as well as specific functional consequences. In case of p63 DNA binding domain (DBD) mutations this could be easily brought about by their disruptive effect towards different sets of target genes. Of the five EEC syndrome hotspot mutations only p.R279 and p.R304 are directly involved in the DNA binding, and possibly lead to loss of binding (26). Other frequently mutated residues (p.R204, p.R227 and p.R280) do not directly interact with DNA and can have gain of binding probabilities on more restricted set of target gene regulatory elements. This is in agreement with results from our unpublished transactivation studies, which demonstrate that hotspot mutants behave differently towards expression of target genes. Secondly, it is also possible that p63-mutant proteins have acquired new functions (gain-of-functions effects of the mutations) that exhibit mutation-specificity. Indeed, such gain-of-function activities have been demonstrated for analogous hot-spot mutations in *p53* (27,28). Thirdly, genetic background in patients, single nucleotide polymorphisms (SNP) in modifier genes might play a role leading to different functional consequence and different clinical phenotype, even for identical primary *p63* mutations. Expression profiling studies on patients of different p63-associated syndromes and on several patients of each hot spot mutation would give insight to the genes that are affected due to each syndrome and each single mutation, and would provide information for explaining the variable clinical appearance.

The underlying disease mechanism in p63-associated syndromes is not yet fully understood. A *p63* gene containing deletion of the long arm of chromosome 3 did not lead to a phenotype similar to *p63* (29,30), and heterozygous *p63* KO-mice did not present a phenotype that resembles p63 syndrome (16,31).

Table 1. Possible disease mechanisms in p63-associated diseases.

	Dose-dependent			
	Loss of function	**Haplo-insufficiency**	**Gain of function**	**Dominant negative**
Hereditary	Recessive conditions	Dominant conditions	Dominant conditions	Dominant conditions
Evidence against		- Del 3q27-qter* and 3q27.3q29* did not lead to p63-syndrome phenotype (29,30) - *p63*$^{+/-}$ KO did not lead to p63-syndrome phenotype - No *p63*-deletions found in p63-patients (unpublished) - Stop mutations in *p63* do not cause NMD		
Evidence for			- ADULT syndrome mutation p.R298 (Chapter 4) - Protein accumulation may indicate that protein is longer active (unpublished) - *p53* hotspot mutants show gain of function (27,28)	- p63 molecule active as tetramer - Shown for AEC/RHS *p63* gene 5' mutations (Chapter 6)

* Includes *p63* gene

These observations provide evidence against dose-dependent mechanism such as haploinsufficiency to cause p63-associated disease phenotype. Moreover, absence of *p63* gene deletions in patients having p63-disease phenotype (our own unpublished data) supports the data that p63-disease is not caused by a missing *p63* allele. (Table 1)

Mechanisms for dominantly inherited conditions such as gain-of-function or dominant-negative are both putative molecular disease mechanisms for p63-linked diseases. Mutant p63 protein might have gained a stronger binding to a regulatory element or to a co-factor involved in transcription regulation leading to enhanced target gene regulation. It is possible that the mutant p63 protein has acquired a completely new function because of new protein conformation i.e. binding to a completely new target gene promoter activating its transcription. Conversely, the mutant allele may have gotten novel dominant-negative activities acting antagonistically against the wild type p63 inhibiting its ordinary function. An example of this are the 5' end mutations in the *p63* gene in AEC/RHS syndromes leading to ΔΔNp63α protein (Chapter 6) that exhibits dominant-negative effects towards the wild type ΔNp63α protein. Dominant negative mutations can be loss of binding mutations that do not allow the p63-tetramer complex to bind to its target region. It is unknown how many mutant protein molecules are needed to inactivate the p63-tetramer function. Our preliminary unpublished results suggest, however, that in some cases dominant negative effect might also be partial. Thus, mutants show reduced binding to regulatory region, but they

do not fully inactivate the functional wild-type protein (unpublished results). Whether this is relevant to the target gene activation has to be investigated in further studies. The p63 pathogenesis seems to be very complex, and it is possible that p63 phenotypes are caused by different mechanisms on molecular level depending on the disease causing mutation (Table 1).

8.3 Resolving p63 regulatory networks and target genes in ED

To resolve the role of p63 in ectodermal development and pathogenesis in different p63-related syndromes, a better picture should be established of regulatory networks, up- and downstream of p63. p63 is a master regulator of a wide variety of cellular processes: epithelial development and maintenance, morphogenesis, proliferation, senescence, tissue regeneration, cell adhesion and cell death (2,6,32-35). Several genome-wide binding and expression studies have been conducted to discover new p63 target genes (6,34,36-38). Unfortunately, the number of potential target genes is so high that it is impossible to select those genes that might contribute to the p63-phenotype. In addition, the overlap of the genes resulted from these studies is difficult to determine because of different cellular systems and techniques.

The approach we have chosen to find p63 regulatory networks and disrupted target genes in different p63-syndromes is a combination of genome-wide DNA binding profiling by using Chromatin-immuno-precipitation-sequencing (ChIP-seq) and gene expression studies in human primary keratinocytes (Chapter 7). This is the first study that uses a relevant human tissue type for the p63 regulatory element search and combines it with functional data originating from expression profiling in patients keratinocytes. Our genome-wide DNA binding assay resulted in more than 11,000 p63 binding sites in the human genome, which is about two times as much as Yang et al detected in cervical carcinoma cell line (6). About 2,500 of these binding sites are the same amongst that study and ours. A high percentage (93%) of the binding sites we detected contain at least one p63 binding motif, which suggests for a direct binding. Our data propose that p63 can be bound to its specific consensus sequence without having transcriptional consequences. Thus, the binding of p63 to its binding site is not necessarily specific for a certain tissue or developmental stage. Other factors are needed in addition to p63 to regulate the initiation of the transcription of a target gene. Presumably these co-factors are specific for tissue type and developmental stage, and are the "final regulators" of p63 dependent transcription. However, p63 regulated transcription can also be controlled by post-translational modifications either by inhibiting the DNA binding itself or the binding of the co-factors.

p63 signaling pathways and target genes in EEC syndrome

So far p63 has been linked to several signaling pathways and processes that are important in ectodermal development, such as BMP, Notch, FGF and NFkB (12,39-41). It is likely that p63 as a transcription factor does not have its own 'p63 signaling pathway', but instead is a crucial regulating molecule that is involved in a cross-talk between other pathways involved in the epithelial development and limb morphogenesis. Components of some above mentioned signaling pathways have been reported to be affected either in *p63*-deficient mice or *in vitro p63*-mutant cellular studies (1,12,13,39,40,42-44). Yet the involvement of major signaling pathways (BMP, Notch, FGF, NFκB) in human p63-linked patients and/or p63-disease pathogenesis *in vivo* has not been dem-

onstrated. In our expression profiling study with EEC syndrome patients' keratinocytes (Chapter 7) we have not detected differential expression for any of the genes that have been reported in the above mentioned pathways. This can be due to tissue and development-specific gene or isoform expression of p63 or other co-factors, or because keratinocytes cannot fully represent all in vivo features of skin pathology. However, members of important developmental pathways without a known association to *p63* during ectodermal development such as Transforming growth factor β (TGFβ) and Wingless type β-catenin (WNT) signaling pathways were abnormally regulated in EEC syndrome patients in comparison to controls (unpublished data). From the TGFβ-pathway we detected *Transforming growth factor β induced protein (TGFβI), Inhibitor of DNA binding 3 (ID3)* and *Decorin (DCN)*. From the WNT signaling pathway we detected *Frizzled-7* and *Kremen-2*. Some of these genes have already been associated with human diseases, such as *TGFBI* and *Decorin* mutations in corneal dystrophies that are also frequently seen in p63-associated disorders (45,45,46). The association into TGFβ- and WNT-signaling indicates that p63 can regulate genes from these pathways in epidermal cells.

In our expression profiling assay performed on EEC patients keratinocytes 328 genes were significantly abnormally expressed compared to controls ($P < 0.05$) (Chapter 7). Sixty percent (198 out of 328) of these abnormally expressed genes in EEC patients have a p63 binding site in a gene or within a 25 kb distance of the gene. These 198 genes could be direct target genes of p63 with possible link to p63 disease phenotype. Other abnormally expressed genes without a consensus binding motif within a 25 kb distance of the corresponding gene are most likely not direct targets of p63, but probably influenced through the direct targets of p63. However, some of the abnormally expressed genes can also be regulated by long-distance p63 binding site, which are more difficult to link to the gene(s) which are under its control.

A significant number of the 198 genes that we presume to be direct targets of p63 and abnormally expressed in EEC patients were developmental genes (Chapter 7). Interestingly genes involved in ectodermal, organ, system and tissue development, as well as genes involved in regulation of biological process and cell cycle were significantly enriched in our comparative expression analyses ($P < 0.005$). Also the established OMIM disease database search of the 198 putative p63 direct target genes resulted in 32 known disease genes (55 disease descriptions). It is very convincing that those 32 genes are involved in p63 disease phenotype, since the features associated with those genes are linked to epithelium and adhesion, and are often seen in p63 patients (ED, orofacial clefting, hearing impairment and corneal dystrophy) and genodermatoses. For example, mutations in *Forkhead box E1 (FOXE1)* have been linked to Bamforth-Lazarus syndrome (OMIM 241850), which combines ectodermal dysplasia (ED) with cleft/lip palate (CLP) syndrome. Mutations in *Distal-less homeobox 3 (DLx3)* have been linked to Tricho-osseous syndrome (OMIM 190320) which is an ED syndrome with bone anomalies (47,48). In addition, mutations in *Collagen XVII type Alpha-1 (COL17A1)* and *Laminin β3 (LAMB3)* have been linked to Herlitz and/or non-Herlitz type of junctional epidermolysis bullosa (OMIM 226700 and OMIM 226650) and *Keratin 2* to Ichtyosis bullosa of Siemens (OMIM 146800).

Interestingly, we detected a strong p63 binding with a p63 consensus sequence in the known Split hand/foot malformation 1 (SHFM1) locus on chromosome 7. This region contains two interesting candidate genes *Distal-less homeobox 5 (DLX5)* and *Distal-less homeobox 6 (DLX6)* that did not show changed gene expression in keratinocytes of EEC patients. However, our data suggest that this locus is a regulatory region that controls expression of *DLX5* and *DLX6* genes during limb development (Chapter 7). Why then was the expression of these genes not changed in the EEC

patients although they all exhibit limb abnormalities? One obvious explanation is that keratinocytes may not be the appropriate cell type for investigating expression of genes that are important in limb development. The intrinsic implication of this explanation is that p63 can be bound to its regulatory regions although it does not necessarily have transcriptional consequences. To trigger the transcription initiation of a target gene some probably tissue-specific p63 binding co-factors are needed. This is an intriguing and new observation for p63, which demonstrates that expression of wild-type transcription factor that is bound to its regulatory element of a target gene might not be active in transcription.

Genetic modifiers involved in p63 pathogenesis

While expression studies like the one we have conducted can provide important information about genes that are disrupted by *p63* hotspot mutations, it will not resolve the clinical variation that has been observed within one hotspot group. This is the variation that can also be seen within a family that is carrying the same single pathogenic mutation. The reason why some family members are more severely affected than others can be due to environmental factors as well as genetic modifiers, such as polymorphism and rare DNA variant elsewhere in the genome i.e. in p63 regulatory elements or in the *p63* gene itself. Genetic modifiers of the p63 phenotype have not been detected yet. To obtain sufficient genetic power to uncover genetic modifiers large numbers of patients carrying the same mutation are needed. Unfortunately, most of the p63-linked diseases are sporadic cases or have been found in small families and are therefore not suitable for this kind of study. However, a large Limb mammary syndrome family with 27 affected patients could represent a useful family for testing the modifier of orofacial clefting, since only six patients of 22 are known to have cleft palate/bifid uvula (49). Also other genes that are known to be involved in non-syndromic orofacial clefting are interesting candidate genes for modifying effects, such as *MSX1*, *Poliovirus receptor like 1 (PVRL1)* and *SUMO1 (50-52)*. A very interesting candidate is also *Interferon regulatory factor 6 (IRF6)*. SNPs in this gene and recently also in the regulatory region of this gene have been linked to the non-syndromic type of orofacial clefting (53,54). Moreover, studies from our group show that this regulatory region is also a direct binding region for p63, and that the *IRF6* gene is down-regulated in the EEC patients that all have cleft lip and palate (Chapter 7 and manuscript under review). In the near future we will test the putative association between this SNP in the *IRF6* binding region and our EEC patients with orofacial clefting, to determine whether this *IRF6* regulatory SNP is associated with increased risk for CLP in p63-syndromes.

Target genes involved in protein degradation

The identification of direct or indirect target genes by expression profiling is not only important to gain insight into ED pathology, but may also shed light onto disease-linked metabolic/biochemical abnormalities observed in patients. In our unpublished studies on primary keratinocyte cultures derived from p63-linked patients we have detected a clear accumulation of ΔNp63α protein in EEC syndrome patients carrying p.R204W, p.R279H, p.R280C and p.R304W mutations. The ΔNp63α protein accumulation appears to be specific for DNA binding domain mutations observed in EEC syndrome patients, since it has not been detected in keratinocyte cultures derived from LMS (p.G76W), RHS (p.Q11X) or AEC (p.T533P and p.G561D) syndrome patients (Fig. 1 and data not shown). It is currently not clear why only mutations in the DNA binding domain give rise to p63 protein accumulation and whether this phenomenon is significant to the pathology of EEC syndrome.

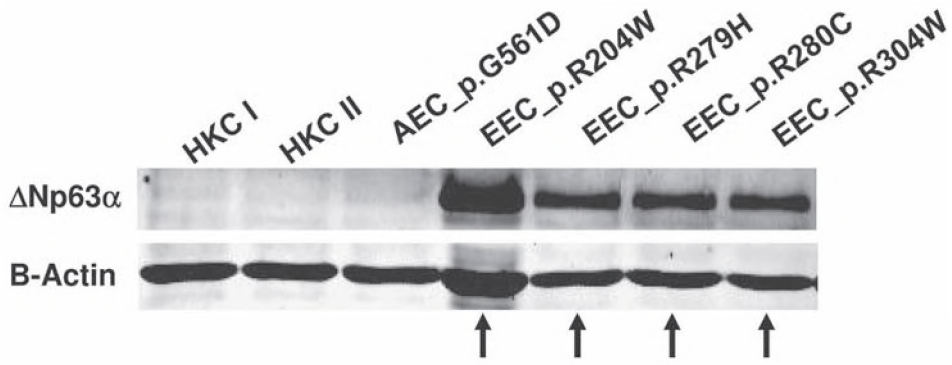

Figure 1. ΔNp63α protein accumulation in EEC patients. ΔNp63α protein accumulates in human primary keratinocytes derived from four different EEC patients (arrows), but not in control keratinocytes or keratinocytes derived from a patient carrying AEC syndrome mutation

P63 family member p53 is known to be degraded by ubiquitin-mediated proteasomal degradation. Mouse double minute 2 homolog (MDM2) is included in the degradation process and forms an auto-regulatory negative feedback loop with p53 (Fig. 2A) (55,56). Possibly, a similar negative feedback loop exists for p63, which could be involved in p63 accumulation in EEC syndrome patients. Indeed, our unpublished data revealed that ΔNp63α protein levels in keratinocytes from EEC patients accumulate, whereas the mRNA levels remained similar to controls, suggesting the involvement of post-translational regulation of the ΔNp63α protein levels. Therefore, we hypothesized that the mutated DNA binding domain of p63 in EEC syndrome patients might not be able to bind and regulate (activate or inactivate) a candidate ubiquitin ligase and therefore its own degradation might be prevented causing p63 accumulation in the cells derived from the EEC patients (Fig. 2B). Recently, E3 ubiquitin ligase NEDD4-like ubiquitin protein ligase ITCH and ubiquitin-like protein SUMO-1 have been shown to directly interact with p63α and regulate p63α protein stability (57-60,60,61,61). We have established that expression of ITCH and SUMO1 is not decreased in keratinocytes of EEC syndrome patients (unpublished data). This result is in agreement with the observation that EEC mutations in the p63 DBD are not involved in the direct binding of ITCH or SUMO-1 (60,61).

Interestingly, our expression array experiment (Chapter 7) showed significant up-regulation ($P \leq 0.05$, fold change approximately +1.3) of U-box type E4 ubiquitin ligase UFD2a (alias UBE4B) in all three EEC patients in comparison to controls. UFD2a has been shown to interact and stabilize ΔNp63α preventing its ubiquination (59). Therefore the increased amount of UFD2a mRNA in EEC patients could increase the steady state level and half-life of ΔNp63α in EEC syndrome keratinocytes. In this model, wild type p63 would repress *UFD2a* expression and the mutated p63 would increase the UFD2a transcription by decreased binding to the *UFD2a* promoter region (Fig. 2C). In agreement with this model, we have found a p63 regulatory region in the close vicinity of the UFD2a gene suggesting that it is a putative direct ΔNp63α target (Chapter 7). To investigate whether UFD2a up-regulation is involved in ΔNp63α protein accumulation in EEC patients has to be investigated by protein-protein interaction and protein accumulation studies in patient derived material.

Candidate genes and regulatory elements for p63-related disorders

The combination of expression, regulatory element and binding data reveal also several genes that might be good candidate genes for ectodermal dysplasia syndromes for which the disease gene is unknown. Multiple genes, whose family members have been linked to ED are differentially regulated in our EEC patients, indicating a potential role for these genes in ectodermal development. For instance *keratins* are generally involved in epidermal development and linked in to different skin diseases and ED, and *Claudin* genes that are involved in formation of tight junctions between epithelial cells might be good candidate genes for ED. *Keratin 33B, Claudin 7* and *Claudin 8* show significantly changed expression in EEC patients. Homeobox genes are involved in embryonic segmental patterning and mutations in several homeobox genes are known to cause ED. We identified *HOXB7* to be significantly changed in EEC patients. Furthermore, the *Lumican* gene is significantly down-regulated in EEC patients. *Lumican* is similar to *Decorin*, which is a proteoglycan interacting with collagen and mutated in corneal dystrophy. Similar to *TGFBI*, disrupted expression of *Lumican* could contribute to the corneal defects that are frequently seen in EEC syndrome. In addition, *Lumican* appears to be a promising target gene to be involved in the development of corneal diseases.

Several ED syndromes have a known genomic localization, but a disease gene or genetic cause has not yet been discovered. Two different studies on consanguineous Pakistani families show hair and nail ED (62,63). Linkage studies revealed homozygous regions in one family on chromosome 12p11.1-q21.1 with a multipoint LOD score of 3 and in another on chromosome 17p12-q21.2 with a multipoint LOD score of 4.4. Both areas are large (37.9 Mb and 21.8 Mb, respectively) containing *keratin* gene clusters, however no mutations in the tested *keratin* genes were found. Interestingly, our expression list has eight genes that locate in the chromosome 12 region and six genes in the chromosome 17 region. None of the genes in chromosome 12 has been linked to hair or nail dysplasia or ectodermal development. Whereas in chromosome 17 there are several potential candidate genes: *Claudin 7, Keratin 31* and *Keratin 33B*. The list of genes that are differentially expressed in the EEC patients may not only help to find undiscovered ED syndrome genes, but also disease genes that have been linked in other single malformations such as split hand/foot or kidney, corneal disease or hearing impairment. Although no genes were found in the currently known and unsolved split hand/foot loci (SHFM1-2 and 5), we found a strong p63 binding and p63 consensus site in the SHFM1 locus in chromosome 7. With help of a novel deletion of this locus in a SHFM patient we were able to redefine the SHFM1 locus, so that previous candidate genes *DLX5* and *DLX6* fall outside of this locus. When adapting the p63 binding data on this locus we identified a p63 regulatory region in the redefined SHFM1 locus to be responsible for SHFM. This p63 regulatory region can now be used for mutation screening in isolated SHFM patients. This result suggests that disease causing defects can also lie outside the coding sequence of a gene of interest and are often missed when investigating only the protein coding sequence or genes in deleted loci.

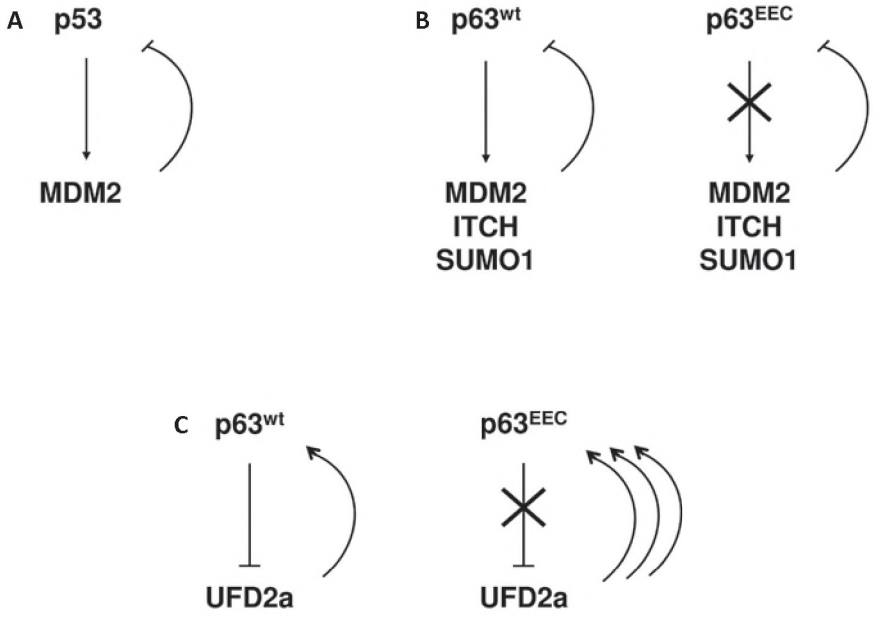

Figure 2. Model of auto-regulatory feedback loop in p53 and p63. A. p53 controls its own degradation through MDM2. **B.** Similar model could be present for p63, which would be disrupted in EEC syndrome patients causing p63 protein accumulation. **C.** In a feedback loop model of p63 and UFD2a, wild type p63 represses UFD2a activation leading to its own stabilization. But, through DBD mutation this repression is lost causing UFD2a up-regulation, which possibly causes p63 accumulation.

8.4 Are there unrecognized pathologies associated with human *p63* mutations?

p63 expression pattern and p63-derived models suggest that *p63* might contribute to other clinical features than ED, limb malformation and orofacial clefting. *p63* has been linked to cellular senescence and aging in heterozygous *p63* knock-out mice (35), that might indicate that *p63* is involved age-related defects. For example, the specific expression of TAp63α in oocytes, its involvement in DNA-damage induced oocyte death (14) and premature ovarian failure (POF) that has been described in a Rapp Hodgkin syndrome family (64) may reflect a role for p63 in female germ line and premature menopause. Corneal defects have also been described in this RHS family (64) and in several of our patients, but it is not known whether this is caused by disrupted cellular senescence and activation of anti-proliferative processes or by failure of stem cell commitment. In addition, early-onset alopecia, skin defects and hearing impairment could be caused by cellular senescence.

TAp63 expression has also been found in the brain (5,18), where TAp63γ is involved in naturally occurring sympathetic neuronal death (18). To our knowledge, there is no evidence for an association between *p63* mutations and the occurrence of neurodevelopmental or neurodegenerative disease. However, unknown defects such as mutation in untranslated region, regulatory element or larger deletion or duplication in the *p63* could play a role in a neuronal disease. TAp63 expres-

sion in heart (5,17), and recently described heart defects (atrial septal defect and arrhythmogenic right ventricular dysplasia/cardiomyopathy) in a RHS (Chapter 6) and an ADULT syndrome patients, respectively, suggest a role for *p63* in heart development (65). To understand whether p63 plays a role in yet unrecognized conditions, such as premature aging, neurodegenerative disorder or heart diseases more detailed studies needs to be performed on physiologically relevant tissue types, since defects in *p63* and pathogenesis might be different in diverse tissues and conditions.

Because *p63* belongs to the *p53* tumor suppressor gene family it has often been linked to tumor development and cancer. A strong p63 accumulation and amplification of *p63* chromosomal locus 3q26-qter has been detected in squamous and basal cell carcinomas indicating an oncogenic function for p63 (66,67). In contrast, the heterozygous *p63*-KO mice study showed loss of heterozygosity in tumors suggesting a role as tumor suppressor gene for *p63* (68). Other studies indicate that ΔNp63 isoforms are able to induce anti-apoptotic genes and repress pro-apoptotic genes, suggesting a role as an anti-apoptotic protein (69,69-71). However, TAp63 isoforms are able to directly activate death receptors and mitochondrial apoptotic pathway indicating a role as a pro-apoptotic protein (19). Taken together, these results indicate that TA and ΔNp63 isoforms have opposite roles in apoptosis and the balance of these two isoforms can be very important in both apoptosis and tumorigenesis. Unlike *p53* mutations, somatic *p63* mutations have rarely been found in human tumors (72,73). Nevertheless, genome-wide association studies in large cohorts from Iceland and the Netherlands revealed a SNP close to the *p63* gene that confers an increased susceptibility for urinary bladder cancer (74). Whether patients with *p63* mutations are more predisposed to tumors is unclear. In the literature only one patient has been reported to carry a *p63* germ-line mutation and a malignant B-cell lymphoma (75). Surprisingly, in our studies we describe patients with a malignant oral squamous cell carcinoma, malignant melanoma and malignant spinocellular epithelioma (Chapters 4, 6 and 7). To determine the incidence of cancer in p63 mutation carriers, we have investigated the medical records of 227 p63-linked patients (Chapter 2) without finding a significant amount of patients carrying a *p63* germ-line mutation and having a cancer. Therefore we cannot conclude that the germ-line *p63* mutations would be causative for the described tumors in the three patients described in Chapters 4, 6 and 7 or that patients with a *p63* germ-line mutation would be more predisposed to a cancer.

8.5 Future visions

Research on p63 and its involvement in ectodermal development and ectodermal dysplasia has progressed very rapidly in the last few years. p63 is now recognized as an important master molecule in ectodermal development and its function has been linked to a variety of cellular functions (1,12,41). A number of direct target genes and regulatory elements have already been described. However, to complete the p63-puzzle with its upstream regulators, p63 interacting proteins i.e. involved in transcription regulation and downstream target genes more research is warranted. Research, that I began in this thesis deals with p63 downstream target genes with a priority to understand the pathogenesis of p63-associated ectodermal dysplasia syndromes. The importance and relevance of these novel target genes in ectodermal development needs subsequent confirmation by other methods, e.g. in mouse models. The last research chapter in this thesis already enabled to find p63 wild-type binding sites in the genome. In the near future this method will be used for keratinocytes derived from p63 syndrome patients, but also for different developmental stages of the epidermis. Future research should also incorporate the differential

properties of the different p63 isoforms, and address the role and expression of less known and understood p63β, p63γ and ΔΔp63 isoforms.

What does our p63 research mean for patients? The importance of p63 research for patients has so far been to facilitate DNA-diagnostic testing and the consequent improvement of genetic counseling. The possibilities for therapeutic intervention are so far limited to cosmetic surgery. The discovery of new direct target genes of p63 and p63 genetic modifiers may have its merits for further improvement of predictive diagnosis as well as for future therapeutic strategies for treatment of p63 patients. If a modifier is involved in creating a certain clinical feature, it can be used to predict the phenotype in prenatal research in the future. Predicting a phenotype with help of genetic modifiers can be complicated, since combinations of different and independent modifiers are likely to account for the final phenotype. However, whole-genome sequencing of large numbers of individuals will soon be a common practice which eventually provides in-depth knowledge about the plasticity of the human genome and its relations to disease. New p63 target genes and regulatory elements are also interesting candidates for other ectodermal dysplasias or other characteristics that belong to the p63 phenotype with a yet undiscovered genetic cause. An excellent example of this is the p63 regulatory element of *DLX5* and *DLX6* genes in SHFM patients (Chapter 7). Finally, if some of the candidate genes or regulatory elements turn out to be novel genetic cause for another ED or disease condition it can be used for establishment of the diagnosis.

Whether a complex *p63* phenotype will be rescued in the future by prenatal therapy is unlikely. Because no single feature in the *p63* phenotype is fully penetrant, it is difficult to determine what to rescue, if the phenotype cannot be predicted on the basis of *p63* mutation. The disease pathogenesis with different p63 isoforms is probably also complex and varied in different tissues that all gene defects could be restored. Nevertheless, if pathogenesis of some single features is established, it could be possible to rescue some certain developmental processes such as hypohidrosis, where sweat gland development or function could be stimulated. Understanding of p63 pathology will not only benefit p63-associated patients but also provide useful information for patients with similar phenotypic features, wound healing and corneal transplantation, since it is expressed in the limbal stem cells and might have a role in regenerative proliferation in the cornea (76-78).

8.6 Concluding remarks

Since the beginning of this thesis, enormous progress has been made in p63 research, including upstream regulation of *p63* and downstream target genes and pathways regulated by p63. This thesis describes a first systematic approach to understand the pathogenesis in p63-associated diseases. Although this work is still at an early stage, it has created a solid base for the search for target genes relevant to ED syndromes, and it will hopefully lead to a better understanding of the complex molecular mechanism of p63-related developmental disorders.

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Summary
Samenvatting

Summary

Mutations in the transcription factor gene *p63* are causative for human developmental syndromes characterized by three main hallmarks: ectodermal dysplasia, limb malformations and orofacial clefting. Five different dominantly inherited human syndromes and two non-syndromic conditions have been linked to *p63* gene defects. The syndromic conditions: Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndrome (EEC), Ankyloblepharon-ectodermal defects-cleft lip/palate (AEC), Limb mammary syndrome (LMS), Acro-dermato-ungual-lacrimal-tooth syndrome (ADULT) and Rapp-Hodgkin syndrome (RHS), all show overlapping features of the three main disease characteristics. In contrast, isolated split hand/foot malformation (SHFM4) and Non-syndromic cleft lip/palate (NSCL) only show one of the three main hallmarks. An overview of the p63-disease associated phenotypes and known causative gene defects are presented in **Chapter 2**.

EEC syndrome is the most common p63-associated condition, since approximately 60 percent of all *p63* mutations have been identified in EEC syndrome patients. In **Chapter 3** a population of 227 clinically described patients carrying a *p63* mutation is presented. This overview demonstrates a large variation in expressivity between p63-syndromes, within a single syndrome and even within patients carrying the same *p63* mutation (even in one family). EEC mutations are mainly missense mutations clustered in the DNA binding domain, and almost 90 percent of all EEC patients carry one of the five hot spot mutations. ADULT syndrome has also been linked to mutations in the DNA binding domain **(Chapter 4)**. Mutations found in AEC and RHS cluster in the amino-terminal transactivation domain (TA/TA2) or in the carboxy-terminal domains (Sterile alpha motif domain, SAM and Transcription inhibitory domain, TI) **(Chapter 5** and **Chapter 6)**. A specific clustering for other p63-associated conditions has not yet been observed.

P63 encodes at least six isoforms through two different promoters and three differential 3' end splicing routes of the gene. ΔNp63α is the only isoform that has been detected in human primary keratinocytes, and is likely the most important isoform in ectodermal development **(Chapter 6** and **Chapter 7)**. Consistent with this is the specific mutation clustering in the ΔNp63α isoform in the AEC and RHS syndrome patients that also have the most striking ectodermal defects **(Chapter 5** and **Chapter 6)**. So far, the role of other p63 isoforms in p63-linked diseases remains unclear.

P63-associated diseases are inherited by autosomal dominant matter. The disease mechanism is not fully deciphered, but there is evidence that mutations in p63 have either gain-of-function and/or dominant negative effects depending on the mutation **(Chapter 6** and **Chapter 8)**. Due to conformational changes, mutant protein can bind to other combinations of target genes leading to transcriptional activation or lose its binding activity to its enhancer by disabling the wild type p63 function in hetero-tetramer molecule. The complex and variable clinical presentation of p63-associated disorders is likely a result of the variable effect of p63 mutations towards expression of legitimate and illegitimate target genes.

To discover and understand the (differential) regulation of the p63 target genes in patients, we studied RNA expression profiling in keratinocytes originated from three EEC patients with p63 hot spot mutations in the DNA binding domain **(Chapter 7)**. This study showed that genes involved in several developmental categories, regulation of biological processes and cell cycle were significantly differently regulated in patients than in controls. This study also indicated several putative candidate genes for other yet unsolved ectodermal dysplasias. To fulfill the expression

profiling study and to discover p63 direct target genes we performed a genome wide DNA binding study on keratinocytes **(Chapter 7)**. It reveals that transcription factor p63 can bind to more than 10,000 binding sites containing a specific p63 binding motive. P63 binding seems not to be specific for tissue or developmental stage. The specificity for transcriptional regulation by p63 is likely controlled by other factors e.g. binding of tissue-specific co-factors.

There is a lot of new data originated from the expression and genome wide DNA binding profiling experiments. Some of this data still needs to be validated and it will hopefully help us to find out relevant target genes for *p63* disease pathogenesis including understanding of separate phenotypes, such as various ED features, SHFM and CLP.

Samenvatting

Mutaties in het transcriptiefactor gen *p63* leiden tot erfelijke ziektes die gekenmerkt worden door verschillende combinaties van drie hoofdkenmerken: ectodermale dysplasie, afwijkingen van de ledematen en gespleten lip en/of gehemelte. Tot nu toe werden vijf verschillende syndromen met dominante overerving beschreven die door defecten in het *p63* gen kunnen ontstaan. Deze ziektebeelden, Ectrodactyly, ectodermal dysplasia and cleft lip/palate syndroom (EEC), Ankyloblepharon-ectodermal defects cleft lip/palate (AEC), Limb mammary syndroom (LMS), Acro-dermato-ungual-lacrimal-tooth syndroom (ADULT) en Rapp-Hodgkin syndroom (RHS), hebben overlappende klinische genmerken van de drie gemeenschappelijke factoren. Daarnaast zijn er twee niet-syndromale ziektebeelden bekend die het gevolg zijn van dominante mutaties in het *p63* gen: geïsoleerde ectrodactyly (splijthand-splijtvoet) (SHFM) en geïsoleerde lip/gehemelte spleet (NSCL). Een overzicht van de p63-geassocieerde fenotypen en de daarbij gevonden gendefecten is beschreven in **Hoofdstuk 2**.

EEC syndroom is de meest bekende p63-geassocieerde ziekte. Ongeveer 60 procent van de p63 mutaties is gevonden in patiënten met het EEC syndroom. In **Hoofdstuk 3** is een overzicht gemaakt van de klinische verschijnselen van 227 patiënten met een mutatie in het *p63* gen. Deze studie liet zien dat er een enorme variatie is van de klinische verschijnselen die gezien werden bij patiënten met een *p63* mutatie. Deze variatie is het grootst tussen de verschillende "p63-syndromen", maar er is ook aanzienlijke klinische variabiliteit binnen één syndroom en zelfs tussen patiënten met dezelfde mutatie (ook binnen één familie). Deze resultaten suggereren dat aanvullende genetische en/of omgevingsfactoren een modulerend effect hebben op het fenotype van de primaire p63 mutatie.

Ondanks de klinische variabiliteit bij p63-geassocieerde aandoeningen is er ook een duidelijk patroon van genotype-fenotype associaties. Mutaties in EEC syndroom patiënten veroorzaken bijna altijd aminozuur veranderingen in het DNA-bindings domein, en bijna 90% van de EEC syndroom patiënten heeft één van de vijf hot spot mutaties. ADULT syndroom mutaties komen ook voor in het DNA–bindings domein **(Hoofdstuk 4)**. Mutaties bij patiënten met AEC en RHS syndroom clusteren in het amino-terminaal transactivatie domein (TA/TA2) of in de carboxy-terminale domeinen (Sterile alpha motif domein, SAM en Transcriptie inhibitie domein, TI) **(Hoofdstuk 5** en **Hoofdstuk 6)**. De syndroom-specifieke clustering van mutaties vormt een basis voor verder functioneel onderzoek naar de functionele karakteristieken van het p63 eiwit.

Het *p63* gen codeert tenminste zes isovormen als gevolg van het gebruik van twee verschillende promoters en drie alternatieve splicing routes. ΔNp63α is de enige isovorm die basale epitheelcellen van de huid (primaire keratinocyten) gedetecteerd kan worden en is derhalve de meest belangrijke isovorm voor de ectodermale ontwikkeling **(Hoofdstuk 6** en **Hoofdstuk 7)**. Bij AEC en RHS syndromen, die de ernstigste ectodermale afwijkingen van de p63-geassocieerde ziektebeelden hebben zijn de mutaties specifiek in de ΔNp63α isovorm geclusterd. De meeste van deze mutaties verstoren het carboxy-terminale einde van het eiwit dat specifiek is voor de a-isovormen. In mijn onderzoek heb ik ook enkele mutaties geïdentificeerd in het amino-terminale einde dat specifiek is voor de ΔN-isovormen **(Hoofdstuk 6)**. Dankzij deze mutaties heb ik tevens ontdekt dat er een alternatief translatie start codon is voor de synthese van een verkorte vorm van ΔNp63α, welke ook in de afwezigheid van *p63* mutaties wordt geproduceerd. Tot op heden is de rol van de andere p63 isovormen in p63-geassocieerde ziektebeelden onbekend.

P63-geassocieerde aandoeningen hebben een autosomale dominante overerving. Het ziektemechanisme is niet volledig opgehelderd, maar er zijn aanwijzingen dat de mutaties de normale activiteit van het mutante en het niet-mutante eiwit remmen (dominant-negatief effect), en tenminste voor sommige mutaties, ook tot nieuwe activiteiten kunnen leiden ("gain-of-function") **(Hoofdstuk 6** en **Hoofdstuk 8)**. De dominant-negatieve mutaties leiden tot verlies van de rol van het p63 eiwit in de regulatie (activatie en repressie) van transcriptie van specifieke genen. Dit effect wordt veelal teweeg gebracht door verlies van binding van p63 eiwitcomplexen (met mutant en normaal p63 eiwit) op specifieke DNA posities in de nabijheid van p63 target genen. Bij gain-of-function mutaties kunnen veranderingen in de conformatie van het eiwit er voor zorgen dat het mutante eiwit de transcriptie van genen stimuleert die normaal gesproken niet onder controle van p63 staan. Het complexe en variabele klinische beeld in de p63-geassocieerde ziektes komt waarschijnlijk door een variabel effect van de p63 mutaties op de transcriptie van zijn rechtmatige en onrechtmatige target genen.

Om te onderzoeken hoe de expressie van p63 target genen bij patiënten is veranderd, heb ik vergelijkende RNA expressie studies uitgevoerd in keratinocyten van drie EEC syndroom patiënten met een verschillende p63 mutatie en van een aantal controle personen **(Hoofdstuk 7)**. Deze studie laat een aantal genen zien die gemeenschappelijk veranderd zijn in expressie bij patiënten ten opzichte van controles. Deze genen zijn betrokken bij een aantal biologische processen die relevant zijn voor het mutante p63 fenotype: epitheliale ontwikkeling en aspecten van embryonale ontwikkeling. Daarnaast resulteerde deze studie ook tot een aantal kandidaatgenen voor erfelijke aandoeningen die fenotypische overlap vertonen met de p63-geassocieerde syndromen. Om te bepalen of deze gedereguleerde genen directe of indirecte targets van het p63 eiwit zijn werden de bindingsplaatsen voor het p63 eiwit op het gehele humane genoom in kaart gebracht **(Hoofdstuk 7)**. Deze studie onthulde dat transcriptiefactor p63 op meer dan 10,000 bindingsplaatsen in het genoom heeft. Een significant aantal van deze bindingsplaatsen werd gevonden in de nabijheid van de genen die gedereguleerd zijn in keratinocyten van de EEC syndroom patiënten. Dit zijn dus waarschijnlijk directe targets van p63. Daarnaast suggereert het grote aantal bindingsplaatsen dat p63 binding niet specifiek is voor een bepaald celtype of fase tijdens de ontwikkeling. De specifieke regulatie van p63 in transcriptie wordt waarschijnlijk gecontroleerd door andere factoren, zoals binding van weefselspecifieke factoren.

De combinatie van genoom-omvattende bepaling van p63 bindingsplaatsen en expressieanalyse heeft een grote hoeveelheid nieuwe data opgeleverd. Hiermee is inzicht verkregen in moleculaire processen die belangrijk zijn voor het ontstaan van het mutante p63 fenotype en de enorme variatie daarvan. Het is te verwachten dat deze data ook van grote waarde zal zijn voor het identificeren van nieuwe genen en regulatoire elementen die betrokken zijn bij erfelijke aandoeningen met overlappende fenotypes, zoals ectodermale dysplasieën, schisis en splijthand-splijtvoet.

List of
Publications
Curriculum Vitae
Acknowledgement
Dankwoorden
Kiitos

List of publications

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*These authors contributed equally to the work.

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Curriculum Vitae

Tuula Rinne was born on June, 13 1977 in Turku, Finland. She finished Luostarivuori High school (Luostarivuoren lukio, Turku, Finland) in May 1997. In autumn 1997 she started Master's degree program in Biology at the University of Joensuu (Finland) and continued it from 1998 at the University of Turku (Finland). She studied genetics as a major and animal anatomy and physiology, plant physiology and biochemistry as minor. In autumn 2001 she studied biomedical sciences at the University of Surrey, in United Kingdom as an Erasmus exchange student. She finished her studies in University of Turku 2003 after writing her master's thesis entitled "Identifying of a genetic defect in a limb-anomalic family". In summer 2003 she performed an additional internship on functional studies on p63 protein in Dr. van Bokhoven's lab at the Department of Human Genetics, University Medical Centre Nijmegen (UMCN), Netherlands. This internship was very fruitful and continued at the beginning of 2004 with a PhD project in Dr. van Bokhoven's group at the UMCN. The PhD research project was performed to understand the role of transcription factor p63 in p63-linked syndromes. It was initiated in a close collaboration with Prof. dr. Schalkwijk at the Department of Dermatology at the UMCN. This PhD research project was finished in July 2008 and is described in this book. Since September 2008 Tuula is working at the section of DNA diagnostics at the Department of Human Genetics as clinical molecular geneticist in training.

Acknowledgement – Dankwoord – Kiitos

Hans

In 2002 heb ik tijdens mijn stage in Finland een nieuwe mutatie in het p63-gen gevonden. Toen ik functionele studies wilde gaan doen heb ik jou gemaild of ik de p63-constructen van jullie zou mogen gebruiken. Al snel heb je me gevraagd om op jouw lab te komen werken om daar de studies uit te voeren. Zomer 2003 als gastonderzoeker op jouw lab vond ik zo geweldig, dat ik maar "ja" heb gezegd tegen het junior onderzoeker project over p63. Ik ben nog altijd blij dat ik dat toen heb gedaan en alle mooie momenten (werk en privé) mee heb mogen maken. Dus bedankt daarvoor, want zonder jouw uitnodiging was ik hier waarschijnlijk nooit gekomen! Bedankt voor de supervisie van dit onderzoek en de mogelijkheden die je mij hebt gegeven. En natuurlijk was je niet alleen mijn wetenschappelijke begeleider, ook bedankt voor jouw rol als mijn "Nederlandse vader" waarin je in het begin met mij een bankrekening hebt geopend en een Sofi-nummer hebt aangevraagd. Ook heb je toen mijn contract van Nederlands naar Engels vertaald, want in het begin kon ik helemaal geen Nederlands!

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Jo

Mooi dat jij co-promoter bij mijn promotie kunt zijn, anders had ik jou als paranimf gevraagd! Ik vond het heel prettig en motiverend dat jij in de laatste jaren onze p63-groep kwam versterken. Ik had jou er graag al vanaf het begin bij gehad! Bedankt voor alles wat ik van jou heb geleerd en al je uitleg, het snelle reageren en voor het nakijken van dingen. Ik vond het geweldig met jou samen te mogen werken!

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Aan het begin van mijn promotieonderzoek was jij nog bezig met je eigen onderzoek aan p63. Ik vond het leuk om samen met jou te werken en dingen met jou te bespreken. Ik wil je bedanken voor al de discussies, het advies en de hulp die ik toen (en soms nog steeds) van jou heb gekregen. Jammer dat je zo ver weg woont!

Ellen
Vanaf het moment dat ik voor de eerste keer in Nijmegen op het lab kwam heb je mij laten zien hoe ik de "voor mij nieuwe" pipetten moest gebruiken en waar alle spullen opgeborgen waren. In het begin hebben wij nog samen aan het p63-project gewerkt. Je bent altijd iemand gebleven met wie ik graag samen ga lunchen en kletsen. Fijn dat jij mijn paranimf wilt zijn!

Emine
Jij bent als eerste onze kleine p63-team komen versterken toen het Epistem-project is begonnen. Het was heel fijn om een stel "extra handen" te hebben en het was ook erg gezellig om met jou samen te werken. Alle sequenties, celkweekjes, transfecties en luciferase assays waren hard nodig voor dit boek. Dus een grote "dankjewel" voor jouw inzet! Ik ben blij dat jij mijn paranimf wilt zijn!

Ben
Hartelijk dank dat ik met jou samen mocht werken op het gebied van p63-geassocieerde ziektebeelden. En bedankt voor de interesse die je altijd in mijn werk hebt getoond.

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Een hartelijke dank aan Ineke, Miranda, Brenda, Gerard en Wil bij wie ik altijd met mijn administratieve vragen terecht kon. Jullie zijn top!

Mensen van de "oude kantoortuin", Dorien, Jeroen, Ilse, Suzanne, Kostas, Kirsten en Karin, bedankt voor de koffiemomenten op het oranje bankje, alle discussies, jullie advies en de gezelligheid! Alle andere (ex-)collega's van het moleculaire lab op P5 hartelijk dank voor jullie professionele hulp en advies. En bedankt nog voor de leuke en gezellige momenten bij de koffie, op de gang, tijdens het dagje uit, enzovoort. Jullie hebben het ook goed gedaan voor mijn Nederlandse inburgering!

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Dear members of the Epistem project (LSHB-CT-2005-019067), National Foundation of Ectodermal Dysplasias and other collaborators from abroad, thank you for the scientific input during my PhD project. It has been an honour to work with you!

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Sukulaiset, ystävät ja tutut. Sydämellinen kiitos teille tuesta ja kiinnostuksesta väitöskirjaani kohtaan!

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Mummo, Tomppa, Olli ja Hani. Kiitokset myös teille että lomilla sai oikeesti olla lomalla eikä tarvinnut puhella töistä. Toivottavasti tämän projektin jälkeen liikenee myös enemmän aikaa yhteydenpitoon. Olette tärkeitä minulle!

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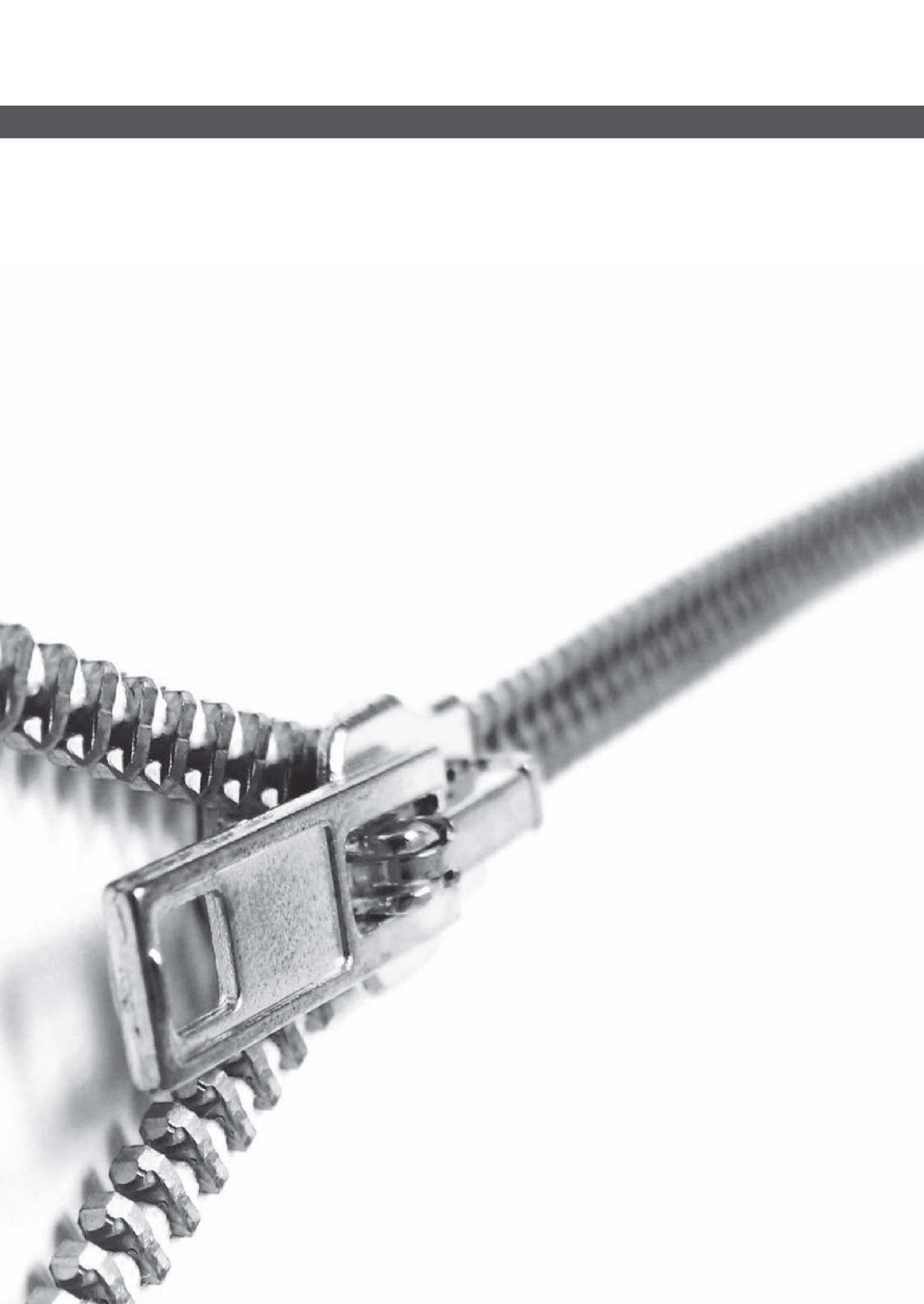


Supplementary Table 5 Chapter 7

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2923928	Fatty acid binding protein 7	FABP7
3455692	Keratin 2	KRT2
3010503	CD36 molecule (thrombospondin receptor)	CD36
3414739	Methyltransferase like 7A	METTL7A
3692999	Metallothionein 1G	MT1G
3255311	Protocadherin 21	PCDH21
3662201	Metallothionein 1H	MT1H
2921402	Solute carrier family 16, member 10	SLC16A10
2806643	Solute carrier family 1, member 3	SLC1A3
3471769	Transmembrane protein 116	TMEM116
3406589	Microsomal glutathione S-transferase 1	MGST1
3928415	Claudin 8	CLDN8
3901041	Thrombomodulin	THBD
3376529	HRAS-like suppressor 3	HRASLS3
3727583	Hepatic leukemia factor	HLF
3753760	Matrix metallopeptidase 28	MMP28
3062665	MGC72080 pseudogene	MGC72080
3067302	Laminin, beta 1	LAMB1
3948640	Fibulin 1	FBLN1
3762083	Distal-less homeobox 3	DLX3
3652424	Eukaryotic elongation factor-2 kinase 4	EEF2K
3761737	Zinc finger protein 652	ZNF652
2468622	Inhibitor of DNA binding 2	ID2
3057755	POM (POM121 homolog, rat) and ZP3 fusion	POMZP3
3917204	Chromosome 21 open reading frame 7	C21orf7
2351687	Chitinase 3-like 2	CHI3L2
3063463	Cytochrome P450, family 3, subfamily A, polypeptide 7	CYP3A7
2451958	Pleckstrin homology domain containing, family A, m. 6	PLEKHA6
2685304	Protein S (alpha)	PROS1
2509988	Hypothetical protein LOC130576	LOC130576
2435005	Selenium binding protein 1	SELENBP1
3972025	Pyruvate dehydrogenase kinase, isozyme 3	PDK3

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_001446	6q22-q23	76,45	0,00019	651,07	0,00008	15,44	0,00224
NM_000423	12q13.13	16,45	0,00189	5,72	0,04108	36,36	0,00051
NM_001001548	7q11.2	9,26	0,00860	7,62	0,03251	22,31	0,00174
NM_014033	12q13.13	5,20	0,00061	5,71	0,00157	7,64	0,00019
NM_005950	16q13	6,43	0,01133	5,48	0,04040	5,58	0,01579
NM_033100	10q22.1-q22.3	3,71	0,02212	8,40	0,00771	4,33	0,01407
NM_005951	16q13	5,40	0,00205	5,89	0,00504	5,02	0,00256
NM_018593	6q21-q22	4,46	0,03393	5,50	0,04863	5,41	0,02137
NM_004172	5p13	3,20	0,00002	3,48	0,00005	5,03	0,00000
BC048796	12q23.13	2,58	0,03236	3,87	0,02026	4,53	0,00447
NM_145791	12p12.3-p12.1	3,34	0,00346	4,58	0,00349	2,77	0,00758
NM_199328	21q22.11	3,24	0,00790	2,89	0,03158	3,41	0,00646
NM_000361	20p11.2	2,19	0,00738	4,13	0,00130	3,09	0,00126
NM_007069	11q12.3-q13.1	3,71	0,00006	2,44	0,00166	3,11	0,00013
NM_002126	17q22	2,05	0,02084	4,39	0,00233	2,80	0,00434
NM_024302	17q11-q21.1	2,92	0,00195	3,38	0,00328	2,90	0,00200
NR_002822	7q21.3	3,50	0,00318	2,55	0,03130	3,03	0,00574
NM_002291	7q22	3,04	0,01098	2,84	0,03612	2,97	0,01204
NM_006486	22q13.31	2,45	0,01395	3,71	0,00735	2,55	0,01158
NM_005220	17q21.33	2,45	0,03091	2,87	0,04007	3,30	0,00970
NM_013302	16p12.1	1,77	0,00699	4,56	0,00015	2,09	0,00203
NM_014897	17q21.32	2,23	0,00230	3,69	0,00063	2,44	0,00137
NM_002166	2p25	2,46	0,01214	2,89	0,01620	3,00	0,00495
NM_012230	7q11.23	2,18	0,01143	1,98	0,04793	3,80	0,00085
AF269162	21q22.3	2,69	0,00266	1,99	0,03545	3,02	0,00154
NM_001025199	1p13.3	2,38	0,00011	2,35	0,00044	2,97	0,00003
NM_000765	7q21-q22.1	1,87	0,00383	2,67	0,00133	2,97	0,00022
NM_014935	1q32.1	2,48	0,00222	2,19	0,01323	2,83	0,00110
NM_000313	3q11.2	1,99	0,01828	2,90	0,00764	2,55	0,00461
AF435957	2q23.2	2,51	0,00972	2,47	0,02723	2,36	0,01320
NM_003944	1q21-q22	1,96	0,03566	2,67	0,02092	2,70	0,00732
NM_005391	Xp22.11	1,71	0,03833	3,32	0,00341	2,23	0,00752

Affymetrix transcript ID	Gene assignment	Gene abbreviation
3662150	Metallothionein 1M	MT1M
3903169	Peroxisomal membrane protein 4	PXMP4
2338719	Nuclear factor I/A	NFIA
3730322	Mannose receptor, C type 2	MRC2
3816699	Zinc finger protein 57	ZNF57
3278057	Coiled-coil domain containing 3	CCDC3
2930243	SAM and SH3 domain containing 1	SASH1
3594986	Testis expressed 9	TEX9
2560625	Transmembrane protein 166	TMEM166
2643901	Protein phosphatase 2	PPP2R3A
2401493	Inhibitor of DNA binding 3	ID3
3050609	Cordon-bleu homolog (mouse)	COBL
2902155	Psoriasis susceptibility 1 candidate 1	PSORS1C1
3899404	Ovo-like 2 (Drosophila)	OVOL2
3826306	Zinc finger protein 85	ZNF85
3808854	Transcription factor 4	TCF4
3376046	Similar to hypothetical protein	LOC221091
3408505	Lymphoid-restricted membrane protein	LRMP
2621881	Hypoxia-inducible factor prolyl 4-hydroxylase	PH-4
3890333	Transcription factor AP-2 gamma	TFAP2C
2376849	Ras association (RalGDS/AF-6) domain family 5	RASSF5
3359224	Achaete-scute complex homolog 2	ASCL2
3290875	Ankyrin 3, node of Ranvier (ankyrin G)	ANK3
3717034	Hypothetical protein LOC400590	LOC400590
2723605	Chromosome 4 open reading frame 19	C4orf19
2969467	Cell division cycle 2-like 6 (CDK8-like)	CDC2L6
2829947	Transforming growth factor, beta-induced	TGFBI
3211938	RAS and EF-hand domain containing	RASEF
3458193	Retinol dehydrogenase 16	RDH16
2605749	Period homolog 2	PER2
2995320	Hypothetical protein DKFZp586I1420	DKFZp586I1420
3968397	WWC family member 3	WWC3

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_176870	16q13	2,27	0,00839	2,21	0,02581	2,61	0,00405
NM_007238	20q11.22	2,01	0,03473	2,33	0,04031	2,51	0,01169
NM_005595	1p31.3-p31.2	1,90	0,00095	3,00	0,00018	1,90	0,00094
NM_006039	17q23.2	2,59	0,00529	2,11	0,03841	1,97	0,02309
EF534355	19p13.3	1,81	0,00363	2,98	0,00053	1,84	0,00317
NM_031455	10p13	1,67	0,04127	2,82	0,00610	2,08	0,01022
NM_015278	6q24.3	2,39	0,00294	1,88	0,03329	2,29	0,00376
NM_198524	15q21.3	2,23	0,00291	2,47	0,00500	1,80	0,01191
BC063016	2p12	2,01	0,00239	2,21	0,00416	2,24	0,00116
NM_002718	3q22.1	1,79	0,01106	2,52	0,00387	2,13	0,00326
NM_002167	1p36.13-p36.12	1,89	0,00849	2,36	0,00644	2,14	0,00380
NM_015198	7p12.1	2,12	0,01679	2,04	0,04875	2,23	0,01282
NM_014068	6p21.3	2,30	0,00912	2,19	0,03046	1,83	0,03345
NM_021220	20pter-q11.23	1,85	0,00628	2,46	0,00313	1,80	0,00774
NM_003429	19p13.1-p12	2,19	0,00090	2,12	0,00361	1,79	0,00398
NM_001083962	18q21.1	1,61	0,01637	2,95	0,00102	1,48	0,03422
NM_203422	11q12.3	1,58	0,02042	2,77	0,00149	1,59	0,01918
NM_006152	12p12.1	1,38	0,04562	3,02	0,00046	1,54	0,01450
NM_177938	3p21.31	1,74	0,00061	1,95	0,00080	2,24	0,00008
NM_003222	20q13.31	1,75	0,00891	2,64	0,00200	1,44	0,04883
NM_182663	1q32.1	1,74	0,03745	1,92	0,04732	2,03	0,01399
NM_005170	11p15.5	1,67	0,04747	2,03	0,03431	1,93	0,01862
NM_020987	10q21	1,76	0,00938	2,31	0,00457	1,55	0,02697
BC062632	17q11.2	1,66	0,04045	2,29	0,01500	1,65	0,04228
NM_001104629	4p14	2,38	0,00001	1,55	0,00161	1,65	0,00023
NM_015076	6q21	1,48	0,00330	2,29	0,00023	1,79	0,00044
NM_000358	5q31	1,84	0,00188	1,87	0,00523	1,84	0,00183
NM_152573	9q21.32	1,84	0,01587	2,09	0,01900	1,63	0,03730
NM_003708	12q13.3	1,90	0,00001	1,80	0,00006	1,83	0,00001
NM_022817	2q37.3	1,92	0,00617	2,02	0,01285	1,58	0,02804
NR_002186	7p15.1	1,33	0,04313	2,17	0,00159	1,89	0,00130
NM_015691	Xp22.32	1,80	0,01856	1,91	0,03211	1,66	0,03290

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2849469	Ankylosis	ANKH
3722355	Rho family GTPase 2	RND2
2435261	RAR-related orphan receptor C	RORC
3139722	Nuclear receptor coactivator 2	NCOA2
3672368	KIAA0182	KIAA0182
3009838	Coiled-coil domain containing 146	CCDC146
3561039	Nuclear factor of kappa light polypeptide gene enhancer	NFKBIA
2429466	Nerve growth factor, beta polypeptide	NGFB
3063501	Cytochrome P450, family 3, subfamily A	CYP3A4
3248470	Chromosome 10 open reading frame 107	C10orf107
3761420	Homeobox B7	HOXB7
2957402	Glutathione S-transferase A3	GSTA3
2614913	Chromosome 3 open reading frame 68	C3orf68
3138204	Cytochrome P450, family 7, subfamily B, polypeptide 1	CYP7B1
3733275	Potassium inwardly-rectifying channel, subfamily J	KCNJ2
3387537	Mastermind-like 2	MAML2
2329266	Zinc finger protein 362	ZNF362
3894194	TBC1 domain family, member 20	TBC1D20
2439554	Absent in melanoma 2	AIM2
3743906	Tumor protein p53	TP53
2970985	TSPY-like 4	TSPYL4
3418214	Methyl-CpG binding domain protein 6	MBD6
2957499	Intestinal cell (MAK-like) kinase	ICK
3264621	Transcription factor 7-like 2	TCF7L2
3726406	Acyl-CoA synthetase family member 2	ACSF2
3600960	Bardet-Biedl syndrome 4	BBS4
3571553	Chromosome 14 open reading frame 43	C14orf43
3061964	Paraoxonase 3	PON3
3926080	BTG family, member 3	BTG3
2994835	Chimerin (chimaerin) 2	CHN2
2697902	Nicotinamide nucleotide adenylyltransferase 3	NMNAT3

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_054027	5p15.1	1,70	0,00062	2,05	0,00044	1,62	0,00106
NM_005440	17q21	1,62	0,02384	1,73	0,03453	1,96	0,00566
NM_005060	1q21	1,70	0,01267	1,77	0,02506	1,81	0,00804
NM_006540	8q13.3	1,73	0,00174	2,09	0,00130	1,44	0,01185
NM_014615	16q24.1	1,66	0,00731	1,79	0,01109	1,70	0,00577
NM_020879	7q11.23	1,49	0,02930	2,05	0,00673	1,60	0,01563
NM_020529	14q13.2	1,57	0,00259	2,16	0,00054	1,40	0,01024
NM_002506	1p13.1	1,93	0,00557	1,63	0,04790	1,55	0,03064
NM_017460	7q22.1	1,35	0,01614	1,76	0,00265	1,98	0,00029
NM_173554	10q21.2	1,61	0,00013	1,44	0,00202	2,03	0,00001
NM_004502	17q21.3	1,59	0,00724	1,76	0,00829	1,70	0,00379
NM_000847	6p12.1	1,70	0,00050	1,83	0,00092	1,52	0,00178
AF086095	3p24.1	1,37	0,02932	2,06	0,00218	1,57	0,00679
NM_004820	8q12.3	1,57	0,00451	2,12	0,00114	1,29	0,04961
NM_000891	17q24.3	1,50	0,01184	1,67	0,01171	1,79	0,00214
NM_032427	11q21	1,48	0,03944	1,92	0,01298	1,51	0,03127
NM_152493	1p35.1	1,35	0,02699	2,03	0,00170	1,50	0,00831
NM_144628	20p13	1,52	0,00454	1,88	0,00182	1,47	0,00653
NM_004833	1q22	2,01	0,00001	1,30	0,00607	1,55	0,00013
NM_000546	17p13.1	1,56	0,01891	1,78	0,01695	1,49	0,02822
NM_021648	6q22.1	1,50	0,04800	1,77	0,03242	1,53	0,03971
NM_052897	12q13.3	1,47	0,01050	1,95	0,00236	1,38	0,02229
NM_016513	6p12.3-p11.2	1,36	0,00662	2,02	0,00034	1,38	0,00528
NM_030756	10q25.2	1,40	0,01045	1,74	0,00309	1,61	0,00207
NM_025149	17q21.33	1,42	0,03093	1,84	0,00836	1,50	0,01765
AY457143	15q22.3-q23	1,47	0,01077	1,89	0,00307	1,37	0,02391
NM_194278	14q24.3	1,41	0,01912	2,01	0,00226	1,32	0,04311
NM_000940	7q21.3	1,66	0,00379	1,45	0,03728	1,61	0,00493
NM_006806	21q21.1-q21.2	1,41	0,04993	1,63	0,03220	1,66	0,01093
NM_004067	7p15.3	1,37	0,00546	1,83	0,00070	1,48	0,00198
NM_178177	3q23	1,27	0,02154	1,45	0,00940	1,96	0,00013

Affymetrix transcript ID	Gene assignment	Gene abbreviation
3192580	Chromosome 9 open reading frame 9	C9orf9
2916716	Proline-rich nuclear receptor coactivator 1	PNRC1
3726211	Pyruvate dehydrogenase kinase, isozyme 2	PDK2
3751590	Coronin 6	CORO6
3822805	Glycoprotein, synaptic 2	GPSN2
3743763	Zinc finger and BTB domain containing 4	ZBTB4
2452069	Phosphoinositide-3-kinase, class 2, beta polypeptide	PIK3C2B
3968512	Chloride channel 4	CLCN4
3268333	HtrA serine peptidase 1	HTRA1
2383356	Chaperone, ABC1 activity of bc1 complex homolog	CABC1
3444009	C-type lectin domain family 7, member A	CLEC7A
2900269	Zinc finger and SCAN domain containing 16	ZSCAN16
3598758	SMAD family member 6	SMAD6
2835021	Prenylcysteine oxidase 1 like	PCYOX1L
3822122	Nuclear factor I/X	NFIX
2657665	Tumor protein p63	TP63
3026599	Tripartite motif-containing 24	TRIM24
3470927	Transient receptor potential cation channel,subfamily V	TRPV4
3377474	Synovial apoptosis inhibitor 1	SYVN1
3497586	Muscleblind-like 2	MBNL2
2359993	cAMP responsive element binding protein 3-like 4	CREB3L4
3651057	Chromosome 16 open reading frame 62	C16orf62
2375706	ATPase, Ca++ transporting, plasma membrane 4	ATP2B4
3741875	Zinc finger, ZZ-type with EF-hand domain 1	ZZEF1
3894637	NSFL1 (p97) cofactor (p47)	NSFL1C
3008376	General transcription factor II	GTF2I
3903089	N-terminal EF-hand calcium binding protein 3	NECAB3
3735346	Hypothetical protein LOC100134934	LOC100134934
3261971	Cyclin M2	CNNM2
3837464	Glioma tumor suppressor candidate region gene 2	GLTSCR2
4018454	Angiomotin	AMOT

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
BC012940	9q34	1,60	0,00142	1,66	0,00322	1,42	0,00588
NM_006813	6q15	1,48	0,00181	1,70	0,00135	1,49	0,00177
NM_002611	17q21.33	1,45	0,00332	1,85	0,00083	1,34	0,00949
NM_032854	17q11.2	1,63	0,00442	1,46	0,03550	1,54	0,00783
NM_138501	19p13.12	1,51	0,01328	1,68	0,01354	1,41	0,02701
NM_020899	17p13.1	1,38	0,00938	1,94	0,00084	1,27	0,03158
NM_002646	1q32.1	1,47	0,02545	1,58	0,03350	1,53	0,01811
NM_001830	Xp22.3	1,54	0,00571	1,43	0,03227	1,61	0,00351
NM_002775	10q26.3	1,43	0,00978	1,55	0,01159	1,58	0,00314
NM_020247	1q42.13	1,43	0,00795	1,68	0,00445	1,43	0,00800
NM_197947	12p13.2	1,59	0,00255	1,47	0,01761	1,46	0,00669
NM_025231	6p22.1	1,38	0,02422	1,55	0,01799	1,57	0,00587
NM_005585	15q21-q22	1,44	0,00334	1,31	0,03269	1,74	0,00037
NM_024028	5q33.1	1,46	0,01147	1,53	0,01843	1,48	0,00957
NM_002501	19p13.13	1,31	0,00433	1,80	0,00025	1,34	0,00265
NM_003722	3q28	1,32	0,04689	1,68	0,01028	1,43	0,01822
NM_015905	7q32-q34	1,31	0,04859	1,71	0,00818	1,37	0,02861
NM_021625	12q24.11	1,29	0,01695	1,37	0,01716	1,70	0,00046
NM_032431	11q13	1,48	0,02271	1,49	0,04811	1,38	0,04566
NM_144778	13q32.1	1,50	0,00158	1,55	0,00345	1,29	0,01450
NM_130898	1q21	1,34	0,02753	1,53	0,01652	1,42	0,01403
AF461052	16p12.3	1,33	0,03209	1,64	0,00938	1,30	0,04319
NM_001001396	1q32.1	1,35	0,01489	1,54	0,00861	1,30	0,02470
NM_015113	17p13.2	1,37	0,00009	1,56	0,00005	1,25	0,00064
NM_016143	20p13	1,30	0,01219	1,55	0,00329	1,31	0,00971
NM_032999	7q11.23	1,37	0,01534	1,38	0,03498	1,40	0,01166
NM_031232	20q11.22	1,50	0,00001	1,23	0,00133	1,42	0,00002
BX648576	17q25.1	1,35	0,00715	1,37	0,01504	1,41	0,00363
NM_017649	10q24.32	1,28	0,02946	1,56	0,00677	1,28	0,02819
NM_015710	19q13	1,22	0,01873	1,58	0,00099	1,33	0,00362
NM_133265	Xq23	1,31	0,00994	1,28	0,03601	1,51	0,00130

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2950515	Vacuolar protein sorting 52 homolog	VPS52
3476457	Nuclear receptor co-repressor 2	NCOR2
3887094	Zinc finger, SWIM-type containing 3	ZSWIM3
3981735	Hypothetical LOC554203	LOC554203
3741997	Ankyrin repeat and FYVE domain containing 1	ANKFY1
3621140	Leucine carboxyl methyltransferase 2	LCMT2
3305081	Collagen, type XVII, alpha 1	COL17A1
3841260	CCR4-NOT transcription complex, subunit 3	CNOT3
2820893	Rieske (Fe-S) domain containing	RFESD
2319560	Ubiquitination factor E4B	UBE4B
3015706	Motile sperm domain containing 3	MOSPD3
3715703	Suppressor of Ty 6 homolog	SUPT6H
3550343	Adenylate kinase 7	AK7
2715476	Chromosome 4 open reading frame 8	C4orf8
3644375	Tuberous sclerosis 2	TSC2
3424379	Chromosome 12 open reading frame 26	C12orf26
3739521	Rabphilin 3A-like	RPH3AL
3528172	T cell receptor alpha locus	TRA@
3294361	Tetratricopeptide repeat domain 18	TTC18
3847252	Scaffold attachment factor B2	SAFB2
3329461	Zinc finger protein 408	ZNF408
2376799	Inhibitor of kappa light polypeptide gene enhancer	IKBKE
3710870	Rho-type GTPase-activating protein RICH2	RICH2
3334304	DnaJ (Hsp40) homolog, subfamily C, member 4	DNAJC4
2321238	PR domain containing 2, with ZNF domain	PRDM2
3346412	Chromosome 11 open reading frame 70	C11orf70
4004044	Dystrophin	DMD
3676134	SplA/ryanodine receptor domain and SOCS box containing 3	SPSB3
3558290	Chromosome 14 open reading frame 124	C14orf124
3254091	Mannose-binding lectin (protein A) 1, pseudogene 1	MBL1P1
3011911	Hypothetical protein FLJ21062	FLJ21062

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_022553	6p2	1,24	0,02069	1,35	0,01453	1,49	0,00124
NM_006312	12q24	1,35	0,00670	1,33	0,02272	1,39	0,00441
NM_080752	20q13.12	1,42	0,00144	1,28	0,02185	1,39	0,00193
BC071776	Xq13.2	1,26	0,02752	1,53	0,00548	1,28	0,02026
NM_016376	17p13.3	1,37	0,00475	1,39	0,01114	1,25	0,02134
NM_014793	15q15.3	1,34	0,00127	1,33	0,00450	1,33	0,00142
NM_000494	10q24.3	1,28	0,01440	1,48	0,00500	1,25	0,02011
NM_014516	19q13.4	1,31	0,01600	1,45	0,01109	1,24	0,03718
NM_173362	5q15	1,43	0,00278	1,35	0,01664	1,22	0,03571
NM_001105562	1p36	1,26	0,00053	1,39	0,00029	1,32	0,00022
NM_001040097	7q22	1,34	0,00110	1,22	0,01929	1,38	0,00067
NM_003170	17q11.2	1,40	0,00125	1,27	0,01914	1,27	0,00623
NM_152327	14q32.2	1,22	0,00612	1,32	0,00382	1,38	0,00055
NM_003704	4p16.3	1,22	0,03936	1,47	0,00652	1,22	0,03806
NM_000548	16p13.3	1,33	0,00547	1,24	0,04454	1,32	0,00623
NM_032230	12q21.31	1,16	0,03366	1,37	0,00423	1,34	0,00175
BC005153	17p13.3	1,32	0,00570	1,25	0,03971	1,29	0,00954
BC070329	14q11.2	1,21	0,04310	1,28	0,03916	1,34	0,00698
NM_145170	10q22.2	1,25	0,02729	1,33	0,02682	1,23	0,03492
NM_014649	19p13.3	1,30	0,00648	1,25	0,03536	1,26	0,01124
NM_024741	11p11.2	1,30	0,00108	1,31	0,00334	1,20	0,00734
NM_014002	1q32.1	1,21	0,00659	1,19	0,02311	1,36	0,00059
NM_014859	17p12	1,23	0,00736	1,37	0,00322	1,16	0,03262
NM_005528	11q13	1,24	0,00676	1,23	0,02138	1,21	0,01164
NM_012231	1p36.21	1,14	0,01947	1,27	0,00388	1,26	0,00127
BC054869	11q22.1	1,23	0,01367	1,26	0,02170	1,17	0,03719
NM_000109	Xp21.2	1,25	0,00286	1,19	0,02395	1,20	0,00658
NM_080861	16p13.3	1,20	0,00455	1,25	0,00506	1,17	0,00814
NM_020195	14q12	1,14	0,01936	1,22	0,00842	1,16	0,01004
NR_002724	10q	1,09	0,00180	1,08	0,00998	1,04	0,03772
NM_001039706	7q21.13	1,04	0,00585	1,06	0,00207	1,09	0,00012

Affymetrix transcript ID	Gene assignment	Gene abbreviation
4026075	Gamma-aminobutyric acid (GABA) A receptor, alpha 3	GABRA3
3841198	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3	NDUFA3
3868518	Hypothetical protein LOC554235	LOC554235
2411642	ATP/GTP binding protein-like 4	AGBL4
2798475	Pleckstrin homology domain containing, family G	PLEKHG4B
2724671	Ras homolog gene family, member H	RHOH
2882196	ATX1 antioxidant protein 1 homolog	ATOX1
3328860	FLJ41423 protein	FLJ41423
3957374	SEC14-like 4	SEC14L4
3050367	Fidgetin-like 1	FIGNL1
3934669	SMT3 suppressor of mif two 3 homolog 3	SUMO3
3230141	Notch homolog 1, translocation-associated	NOTCH1
2378325	SERTA domain containing 4	SERTAD4
3120443	G protein-coupled receptor 172A	GPR172A
2728408	RE1-silencing transcription factor	REST
2360773	Metaxin 1	MTX1
4001654	G protein-coupled receptor 64	GPR64
3217395	Ankyrin repeat and sterile alpha motif domain containing 6	ANKS6
2318637	Vesicle-associated membrane protein 3	VAMP3
3381540	KIAA0280	KIAA0280
2406766	Mitochondrial ribosomal protein S15	MRPS15
3466206	Transmembrane and coiled-coil domain family 3	TMCC3
3883441	Sperm associated antigen 4	SPAG4
2828856	Heat shock 70kDa protein 4	HSPA4
3059464	Sema domain, immunoglobulin domain (Ig)	SEMA3A
2363579	Translocase of outer mitochondrial membrane 40 homolog	TOMM40L
2759654	Actin binding LIM protein family, member 2	ABLIM2
3189714	GTPase activating Rap/RanGAP domain-like 3	GARNL3
3203311	Aprataxin	APTX

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_000808	Xq2	3,04	0,00001	-1,32	0,03012	1,23	0,03802
AK289489	19q13.42	1,45	0,01218	-1,50	0,02307	1,45	0,01224
NM_001024656	19q13.33	1,24	0,00964	-1,23	0,02959	1,23	0,01273
NM_032785	1p33	1,22	0,00690	-1,17	0,04233	1,17	0,01762
NM_052909	5p15.33	1,19	0,02991	-1,24	0,03306	1,22	0,01715
NM_004310	4p13	1,15	0,04483	-1,19	0,04711	1,18	0,02697
NM_004045	5q32	1,21	0,01608	-1,45	0,00231	1,32	0,00304
AK123417	11p11.2	1,21	0,03800	-1,34	0,01974	1,21	0,04275
NM_174977	22q12.2	1,12	0,02080	-1,17	0,01314	1,10	0,04059
BC027856	7p12.2	-1,46	0,02384	1,87	0,00778	-1,40	0,03669
NM_006936	21q22.3	-1,16	0,01074	1,26	0,00468	-1,15	0,01712
NM_017617	9q34.3	-1,28	0,01009	1,41	0,00635	-1,24	0,01756
NM_019605	1q32.1-q41	-1,10	0,02205	-1,12	0,02555	-1,11	0,01233
NM_024531	8q24.3	-1,16	0,00923	-1,26	0,00398	-1,11	0,03549
NM_005612	4q12	-1,23	0,00096	-1,16	0,01391	-1,18	0,00312
NM_002455	1q21	-1,13	0,01619	-1,30	0,00121	-1,15	0,00947
NM_001079858	Xp22.13	-1,17	0,01506	-1,28	0,00560	-1,16	0,02039
BC012981	9q22.33	-1,20	0,01964	-1,29	0,01430	-1,16	0,04158
NM_004781	1p3	-1,17	0,02238	-1,33	0,00539	-1,23	0,00754
D87470	11q13.4	-1,25	0,01122	-1,22	0,04210	-1,27	0,00848
NM_031280	1p35-p34.1	-1,24	0,01264	-1,25	0,02721	-1,29	0,00550
NM_020698	12q22	-1,25	0,01684	-1,33	0,01684	-1,21	0,03163
NM_003116	20q11.21	-1,14	0,03296	-1,52	0,00046	-1,15	0,02602
NM_002154	5q31.1-q31.2	-1,45	0,00007	-1,23	0,00489	-1,14	0,01414
NM_006080	7q21.11	-1,22	0,04289	-1,35	0,02298	-1,24	0,03380
NM_032174	1q23.3	-1,29	0,00903	-1,34	0,01395	-1,19	0,04156
DQ413176	4p16-p15	-1,30	0,00261	-1,34	0,00496	-1,18	0,02158
NM_032293	9q33.3	-1,21	0,00131	-1,36	0,00041	-1,32	0,00019
NM_175073	9p13.3	-1,26	0,00749	-1,37	0,00541	-1,27	0,00619

Affymetrix transcript ID	Gene assignment	Gene abbreviation
3833620	Latent transforming growth factor beta binding protein	LTBP4
2951057	Chromosome 6 open reading frame 1	C6orf1
3705641	Translocase of inner mitochondrial membrane 22 homolog	TIMM22
2330253	ADP-ribosylhydrolase like 2	ADPRHL2
3801492	Ankyrin repeat domain 29	ANKRD29
2933331	Sorting nexin 9	SNX9
2734047	Lung cancer metastasis-associated protein	MAG1
4007617	Pim-2 oncogene	PIM2
2616804	SH3 and cysteine rich domain	STAC
2458513	Transmembrane protein 63A	TMEM63A
3068097	Dedicator of cytokinesis 4	DOCK4
3544141	Iron-sulfur cluster assembly 2 homolog	ISCA2
2364155	U2AF homology motif (UHM) kinase 1	UHMK1
3298924	Multimerin 2	MMRN2
2880679	SH3 domain and tetratricopeptide repeats 2	SH3TC2
3937967	Hypothetical protein	FLJ26056
2835662	MSTP150	MST150
2726072	ATPase, class V, type 10D	ATP10D
3031556	GTPase, IMAP family member 2	GIMAP2
3063083	SMAD specific E3 ubiquitin protein ligase 1	SMURF1
2377094	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	PFKFB2
2703902	Btyrylcholinesterase	BCHE
3937847	Leucine-zipper-like transcription regulator 1	LZTR1
3355056	FAD-dependent oxidoreductase domain containing 1	FOXRED1
3838795	BCL2-like 12 (proline rich)	BCL2L12
3226737	Cysteine conjugate-beta lyase	CCBL1
2834957	Actin filament associated protein 1-like 1	AFAP1L1
3279982	Protein tyrosine phosphatase-like	PTPLA
2757427	Leucine zipper-EF-hand containing transmembrane protein 1	LETM1
3914050	Stathmin-like 3	STMN3

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_001042544	19q13.2	-1,30	0,03220	-1,34	0,04973	-1,27	0,04447
NM_178508	6p21.31	-1,23	0,01059	-1,43	0,00216	-1,28	0,00429
NM_013337	17p13.3	-1,26	0,02456	-1,39	0,01472	-1,29	0,01588
NM_017825	1p34.3	-1,26	0,00504	-1,50	0,00105	-1,20	0,01627
NM_173505	18q11.2	-1,22	0,03377	-1,52	0,00382	-1,24	0,02306
NM_016224	6q25.1-q26	-1,34	0,01053	-1,30	0,03981	-1,35	0,00909
NM_032717	4q21.23	-1,30	0,01600	-1,47	0,00861	-1,23	0,04260
NM_006875	Xp11.23	-1,49	0,00167	-1,34	0,02118	-1,23	0,03178
NM_003149	3p22.3	-1,27	0,03246	-1,58	0,00550	-1,25	0,03859
NM_014698	1q42.12	-1,31	0,04025	-1,48	0,02406	-1,36	0,02620
NM_014705	7q31.1	-1,30	0,01996	-1,58	0,00466	-1,29	0,02246
NM_194279	14q24.3	-1,34	0,00700	-1,39	0,01142	-1,44	0,00241
NM_175866	1q23.3	-1,47	0,00404	-1,35	0,03254	-1,36	0,01106
NM_024756	10q23.2	-1,24	0,03664	-1,43	0,01291	-1,55	0,00163
NM_024577	5q32	-1,36	0,02079	-1,56	0,01163	-1,31	0,03249
AK129567	22q11.21	-1,41	0,00059	-1,69	0,00021	-1,15	0,03299
NM_032947	5q33.1	-1,21	0,02540	-1,79	0,00035	-1,30	0,00618
NM_020453	4p12	-1,51	0,00051	-1,49	0,00207	-1,31	0,00425
NM_015660	7q36.1	-1,46	0,00150	-1,53	0,00267	-1,35	0,00460
NM_020429	7q22.1	-1,42	0,01112	-1,63	0,00739	-1,30	0,03582
NM_006212	1q32.1	-1,50	0,00607	-1,50	0,01749	-1,36	0,02129
NM_000055	3q26.1-q26.2	-1,50	0,00126	-1,34	0,01706	-1,53	0,00094
NM_006767	22q11.21	-1,28	0,02367	-1,57	0,00496	-1,53	0,00212
NM_017547	11q2	-1,44	0,03811	-1,55	0,04470	-1,42	0,04306
NM_138639	19q13.3	-1,40	0,00862	-1,55	0,00800	-1,47	0,00498
NM_004059	9q34.11	-1,52	0,01281	-1,46	0,04618	-1,51	0,01317
NM_152406	5q33.1	-1,54	0,00173	-1,45	0,01070	-1,52	0,00210
NM_014241	10p12.33	-1,62	0,00313	-1,60	0,01033	-1,32	0,03489
NM_012318	4p16.3	-1,46	0,01652	-1,70	0,01072	-1,42	0,02244
NM_015894	20q13.3	-1,38	0,00887	-1,86	0,00112	-1,42	0,00558

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2620538	Leucyl-tRNA synthetase 2	LARS2
2334191	Polo-like kinase 3	PLK3
3403015	Enolase 2	ENO2
4015884	armadillo repeat containing, X-linked 2	ARMCX2
3124537	Cathepsin B	CTSB
3315024	ADAM metallopeptidase domain 8	ADAM8
2794792	Vascular endothelial growth factor C	VEGFC
3719231	Mitochondrial rRNA methyltransferase 1 homolog	MRM1
2618940	Catenin (cadherin-associated protein), beta 1	CTNNB1
2692816	Integrin, beta 5	ITGB5
3420316	High mobility group AT-hook 2	HMGA2
2901913	Tubulin, beta	TUBB
3465248	Lumican	LUM
2522439	Basic leucine zipper and W2 domains 1	BZW1
2655168	YEATS domain containing 2	YEATS2
3168066	Carbonic anhydrase IX	CA9
2401193	Leucine zipper protein 1	LUZP1
3140920	Junctophilin 1	JPH1
3329099	Glycosyltransferase-like 1B	GYLTL1B
2793401	Microfibrillar-associated protein 3-like	MFAP3L
3293724	Chromosome 10 open reading frame 54	C10orf54
3532793	Paired box 9	PAX9
3770505	Usher syndrome 1G	USH1G
3645452	Kringle containing transmembrane protein 2	KREMEN2
3061319	Cyclin-dependent kinase 6	CDK6
2946324	Histone cluster 1, H3d	HIST1H3D
3639601	RGM domain family, member A	RGMA
3743551	Claudin 7	CLDN7
2891015	Tripartite motif-containing 7	TRIM7
3098977	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	LYN
2760869	Heparan sulfate (glucosamine) 3-O-sulfotransferase 1	HS3ST1

Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_015340	3p21.3	-1,68	0,00095	-1,31	0,04685	-1,69	0,00092
NM_004073	1p34.1	-1,28	0,00779	-1,98	0,00013	-1,44	0,00106
NM_001975	12p13	-1,48	0,01427	-1,77	0,00764	-1,46	0,01639
NM_177949	Xq21.33-q22.2	-1,56	0,00673	-1,47	0,03102	-1,69	0,00297
NM_147780	8p23.1	-1,33	0,03046	-1,92	0,00219	-1,52	0,00594
NM_001109	10q26.3	-1,33	0,04523	-1,96	0,00332	-1,48	0,01386
NM_005429	4q34.2	-1,42	0,01708	-1,91	0,00309	-1,46	0,01266
NM_024864	17q12	-1,56	0,00523	-1,67	0,00812	-1,59	0,00424
NM_001904	3p22.1	-1,60	0,00258	-1,86	0,00215	-1,37	0,01723
NM_002213	3q21.2	-1,59	0,01455	-1,68	0,02450	-1,59	0,01488
NM_003483	12q15	-1,46	0,04320	-1,80	0,02020	-1,62	0,01712
NM_178014	6p21.33	-1,59	0,00197	-1,47	0,01422	-1,84	0,00048
NM_002345	12q21.3-q22	-1,55	0,01461	-1,77	0,01230	-1,59	0,01092
NM_014670	2q33	-1,63	0,00377	-1,82	0,00426	-1,50	0,00889
NM_018023	3q27.1	-1,77	0,00024	-1,32	0,02473	-1,92	0,00011
NM_001216	9p13-p12	-1,50	0,03962	-2,05	0,01072	-1,50	0,03935
NM_033631	1p36	-1,52	0,00446	-1,95	0,00143	-1,61	0,00246
NM_020647	8q21	-1,77	0,00959	-1,69	0,03431	-1,64	0,01774
NM_152312	11p11.2	-1,43	0,00418	-2,05	0,00038	-1,63	0,00084
NM_021647	4q32.3	-1,72	0,01579	-1,75	0,03597	-1,67	0,01995
NM_022153	10q22.1	-1,94	0,00236	-1,60	0,03064	-1,61	0,01092
NM_006194	14q12-q13	-1,40	0,02325	-2,30	0,00100	-1,49	0,01147
NM_173477	17q25.1	-1,77	0,00318	-1,96	0,00450	-1,47	0,01919
NM_172229	16p13.3	-1,50	0,02241	-2,25	0,00293	-1,45	0,03190
NM_001259	7q21-q22	-1,50	0,01106	-1,96	0,00306	-1,80	0,00184
BC033095	6p21.3	-1,89	0,00107	-1,48	0,02810	-1,93	0,00092
NM_020211	15q26.1	-1,58	0,02724	-2,13	0,00891	-1,64	0,02011
NM_001307	17p13	-1,68	0,03082	-1,94	0,02980	-1,74	0,02408
NM_203296	5q35.3	-1,98	0,00220	-1,83	0,01189	-1,57	0,01510
NM_002350	8q13	-1,56	0,03897	-2,31	0,00791	-1,58	0,03485
NM_005114	4p16	-1,71	0,01667	-1,91	0,02032	-1,84	0,00967

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2712754	Phosphate cytidylyltransferase 1, choline, alpha	PCYT1A
3554851	Cysteine-rich protein 1	CRIP1
3147985	Low density lipoprotein-related protein 12	LRP12
2453881	Interferon regulatory factor 6	IRF6
3598381	Protein tyrosine phosphatase-like A domain containing 1	PTPLAD1
3450234	Plakophilin 2	PKP2
3536434	Sterile alpha motif domain containing 4A	SAMD4A
3947863	Parvin, beta	PARVB
2401581	UDP-galactose-4-epimerase	GALE
3445574	Guanylate cyclase 2C	GUCY2C
3275922	Protein kinase C, theta	PRKCQ
3091077	Dihydropyrimidinase-like 2	DPYSL2
2403446	Platelet-activating factor receptor	PTAFR
2453793	Laminin, beta 3	LAMB3
3382410	Microtubule-associated protein 6	MAP6
2446047	V-abl Abelson murine leukemia viral oncogene homolog 2	ABL2
2675150	Hyaluronoglucosaminidase 1	HYAL1
3421985	Potassium large conductance calcium-activated channel	KCNMB4
2688499	Zinc finger, BED-type containing 2	ZBED2
3393622	Sodium channel, voltage-gated, type IV, beta	SCN4B
3000953	Uridine phosphorylase 1	UPP1
3569257	Pleckstrin 2	PLEK2
3572209	Placental growth factor, vascular endothelial growth factor	PGF
2520291	Glutaminase	GLS
3033209	Insulin induced gene 1	INSIG1
3435078	WD repeat domain 66	WDR66
3845263	ADAMTS-like 5	ADAMTSL5
2463567	Phospholipase D family, member 5	PLD5
3709417	Arachidonate 15-lipoxygenase, type B	ALOX15B
3610110	Nuclear receptor subfamily 2, group F, member 2	NR2F2

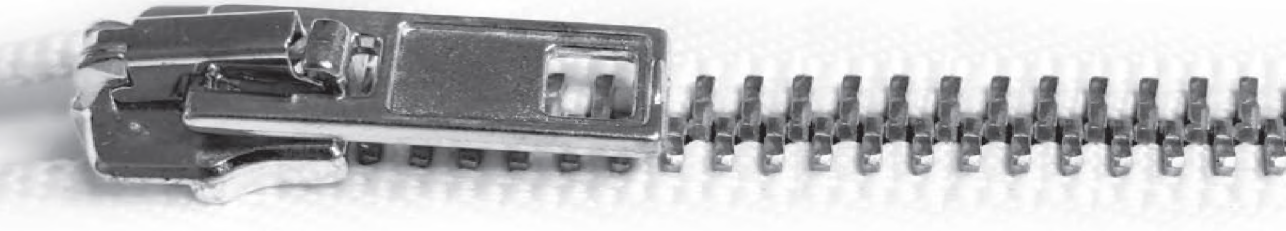
Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_005017	3q29	-1,63	0,00129	-2,48	0,00016	-1,37	0,01040
NM_001311	14q32.33	-1,82	0,00508	-2,02	0,00705	-1,69	0,00906
NM_013437	8q22.2-q23.1	-1,94	0,00595	-1,75	0,03210	-1,87	0,00781
NM_006147	1q32.3-q41	-1,60	0,00619	-2,32	0,00114	-1,67	0,00414
NM_016395	15q22.31	-1,84	0,00154	-2,40	0,00077	-1,37	0,02833
NM_004572	12p11	-1,58	0,04017	-2,06	0,01760	-2,04	0,00679
NM_015589	14q22.2	-1,44	0,02331	-2,62	0,00078	-1,69	0,00503
NM_001003828	22q13.2-q13.33	-1,70	0,03223	-2,35	0,01256	-1,79	0,02256
NM_000403	1p36-p35	-2,09	0,01129	-2,05	0,03244	-1,74	0,03554
NM_004963	12p12.3-p12.1	-1,91	0,01912	-2,10	0,02780	-1,86	0,02225
NM_006257	10p15	-1,76	0,00098	-2,17	0,00064	-1,95	0,00040
NM_001386	8p22-p21	-2,06	0,00414	-2,33	0,00595	-1,50	0,04638
AK292053	1p35-p34.3	-1,59	0,02620	-2,23	0,00700	-2,13	0,00308
NM_001017402	1q32	-1,69	0,03734	-2,60	0,00851	-1,68	0,03799
NM_207577	11q13.5	-1,76	0,04403	-2,43	0,01961	-1,79	0,03971
NM_007314	1q24-q25	-1,53	0,01687	-2,92	0,00062	-1,56	0,01416
NM_007312	3p21.3-p21.2	-2,23	0,00170	-2,34	0,00406	-1,51	0,03342
NM_014505	12q15	-1,90	0,02617	-2,25	0,02641	-1,95	0,02253
NM_024508	3q13.13	-1,68	0,04446	-2,57	0,01078	-1,86	0,02322
NM_174934	11q23.3	-1,87	0,01215	-1,87	0,03127	-2,39	0,00261
NM_003364	7p12.3	-1,82	0,00023	-2,55	0,00007	-1,78	0,00028
NM_016445	14q23.3	-1,74	0,02091	-2,35	0,00906	-2,10	0,00583
NM_002632	14q24-q31	-1,76	0,01857	-2,66	0,00466	-1,78	0,01722
NM_014905	2q32-q34	-1,74	0,04935	-2,54	0,01693	-1,92	0,02686
NM_198336	7q36	-2,43	0,00175	-2,05	0,01404	-1,76	0,01472
NM_144668	12q24.31	-1,89	0,00393	-2,26	0,00371	-2,08	0,00197
NM_213604	19p13.3	-2,14	0,01434	-2,15	0,03512	-1,97	0,02284
NM_152666	1q43	-2,17	0,00289	-2,37	0,00535	-1,82	0,00984
NM_001141	17p13.1	-1,49	0,03450	-2,59	0,00208	-2,38	0,00099
NM_021005	15q26	-2,39	0,00025	-2,16	0,00167	-2,02	0,00080

Affymetrix transcript ID	Gene assignment	Gene abbreviation
2413203	Low density lipoprotein receptor-related protein 8	LRP8
4054427	Gap junction protein, beta 4	GJB4
3041816	Deafness, autosomal dominant 5	DFNA5
3352948	Sortilin-related receptor, L(DLR class) A repeats-containi	SORL1
3464276	Solute carrier family 6, member 15	SLC6A15
2523045	Frizzled homolog 7	FZD7
3690154	Neuropilin (NRP) and tolloid (TLL)-like 2	NETO2
3353335	Ubiquitin associated and SH3 domain containing, B	UBASH3B
2344393	Protein kinase, cAMP-dependent, catalytic, beta	PRKACB
4007437	Solute carrier family 38, member 5	SLC38A5
2925590	Transmembrane protein 200A	TMEM200A
3976639	Porcupine homolog	PORCN
2635906	Pleckstrin homology-like domain, family B, member 2	PHLDB2
2587790	G protein-coupled receptor 155	GPR155
2686023	Discoidin, CUB and LCCL domain containing 2	DCBLD2
3070183	Aminoadipate-semialdehyde synthase	AASS
3726691	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	ABCC3
3378541	Pyruvate carboxylase	PC
3465274	Decorin	DCN
3467315	IKK interacting protein	IKIP
3414351	Amiloride-sensitive cation channel 2, neuronal	ACCN2
3756896	Keratin 33B	KRT33B
3473524	Nitric oxide synthase 1	NOS1
3121198	Chromosome 8 open reading frame 42	C8orf42
3549605	KIAA1622	KIAA1622
2774971	Anthrax toxin receptor 2	ANTXR2
2540157	Ornithine decarboxylase 1	ODC1
3468743	5'-nucleotidase domain containing 3	NT5DC3
3758510	Ets variant gene 4	ETV4
3464405	Peptidylglycine alpha-amidating monooxygenase COOH-termina	PAMCI
3864551	Plasminogen activator, urokinase receptor	PLAUR

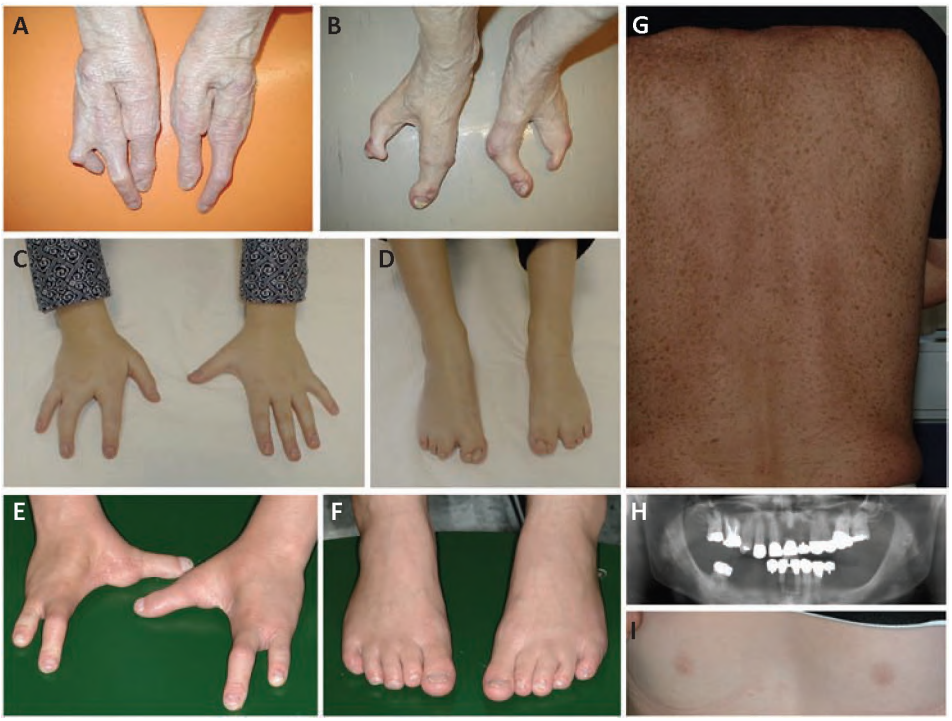
Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_004631	1p34.3	-2,08	0,00400	-2,29	0,00671	-2,23	0,00253
NM_153212	1p34.3	-1,52	0,04644	-2,64	0,00379	-2,50	0,00157
NM_004403	7p15	-2,20	0,01731	-2,45	0,02668	-2,13	0,02038
NM_003105	11q23.2-q24.2	-1,53	0,03025	-2,95	0,00132	-2,31	0,00146
NM_182767	12q21.3	-2,25	0,00872	-2,21	0,02553	-2,38	0,00634
NM_003507	2q33	-2,01	0,00915	-3,29	0,00220	-1,58	0,04727
NM_018092	16q11	-2,36	0,00268	-1,76	0,04290	-3,04	0,00071
NM_032873	11q	-1,49	0,02941	-3,32	0,00053	-2,40	0,00082
NM_182948	1p36.1	-2,25	0,00599	-2,58	0,00876	-2,39	0,00436
NM_033518	Xp11.23	-2,20	0,00817	-3,03	0,00505	-2,02	0,01340
BC044246	6q23.1	-2,07	0,01678	-2,07	0,04064	-3,19	0,00195
NM_022825	Xp11.23	-2,16	0,01187	-2,77	0,00972	-2,51	0,00531
NM_145753	3q	-1,93	0,03428	-2,83	0,01422	-2,84	0,00495
NM_001033045	2q31.1	-2,95	0,00207	-2,60	0,01113	-2,45	0,00514
NM_080927	3q12.1	-2,55	0,00691	-2,85	0,01183	-2,62	0,00605
NM_005763	7q31.3	-2,90	0,00494	-2,30	0,03658	-2,93	0,00470
NM_003786	17q22	-2,32	0,03146	-3,00	0,02807	-2,83	0,01380
NM_001040716	11q13.4-q13.5	-2,93	0,00075	-2,85	0,00288	-2,39	0,00222
NM_001920	12q21.33	-3,09	0,00155	-2,66	0,00950	-2,45	0,00480
NM_153687	12q23.1	-2,86	0,00183	-2,54	0,00995	-2,84	0,00191
NM_020039	12q12	-2,74	0,00444	-2,81	0,01159	-2,70	0,00472
NM_002279	17q12-q21	-2,64	0,00099	-3,28	0,00118	-2,86	0,00065
NM_000620	12q24.2-q24.31	-3,10	0,00968	-2,99	0,02887	-2,81	0,01421
NM_175075	8p23.3	-3,22	0,00095	-1,91	0,03895	-3,89	0,00043
NM_058237	14q32.13	-2,71	0,00269	-2,85	0,00651	-3,55	0,00079
NM_058172	4q21.21	-3,32	0,00352	-2,96	0,01589	-2,91	0,00605
AK292352	2p25	-2,26	0,02094	-4,42	0,00420	-2,66	0,00965
NM_016575	12q22-q23.1	-2,52	0,01621	-3,47	0,01244	-3,54	0,00396
NM_001986	17q21.31	-3,03	0,01220	-3,35	0,02228	-3,44	0,00755
NM_005447	12q21.31	-2,77	0,01220	-4,24	0,00737	-3,03	0,00845
NM_002659	19q13	-2,12	0,03007	-5,67	0,00211	-2,97	0,00647

Affymetrix transcript ID	Gene assignment	Gene abbreviation
3181374	Forkhead box E1	FOXE1
2496907	Interleukin 1 receptor, type II	IL1R2
2571510	Interleukin 1, beta	IL1B
2748605	Lecithin retinol acyltransferase	LRAT
3485674	Cyclin A1	CCNA1
2618620	Ectonucleoside triphosphate diphosphohydrolase 3	ENTPD3
3486956	Chromosome 13 open reading frame 15	C13orf15
3638411	Rh family, C glycoprotein	RHCG
2787459	Inositol polyphosphate-4-phosphatase, type II	INPP4B
2938972	Serpin peptidase inhibitor, clade B, member 1	SERPINB1
3396593	Fasciculation and elongation protein zeta 1	FEZ1
2359483	Small proline-rich protein 4	SPRR4
2501120	Interleukin 1 family, member 9	IL1F9
4013549	Integral membrane protein 2A	ITM2A
2434609	Cathepsin K	CTSK
4008427	Nudix (nucleoside diphosphate linked moiety X)-type motif	NUDT11
2345816	Guanylate binding protein family, member 6	GBP6
2779302	Alcohol dehydrogenase 7	ADH7
3568603	Glutathione peroxidase 2	GPX2
3756928	Keratin 31	KRT31

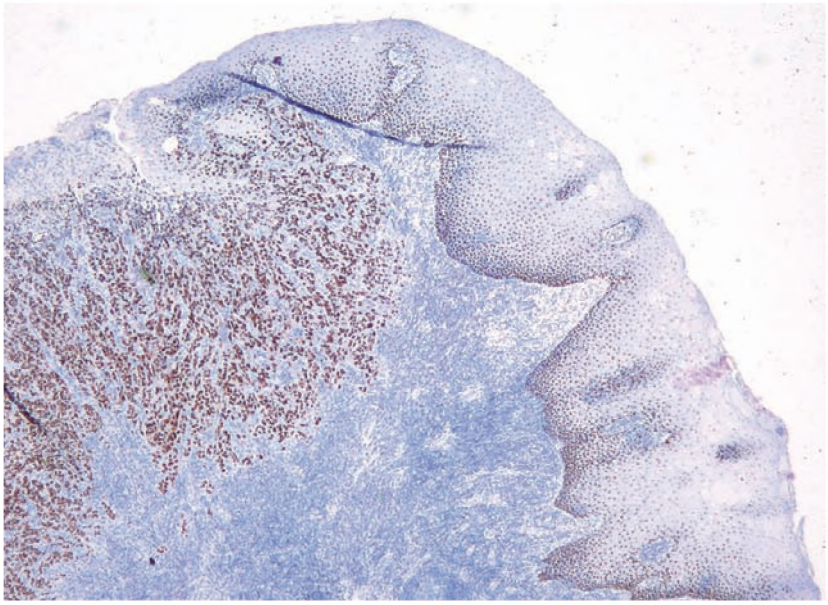
Reference sequence	Chromosomal location	Fold Change R204W	p-value (R204W)	Fold Change (R279H)	p-value (R279H)	Fold Change (R304W)	p-value (R304W)
NM_004473	9q22	-2,97	0,00252	-4,74	0,00136	-3,22	0,00177
NM_004633	2q12-q22	-2,69	0,03247	-4,70	0,01415	-3,71	0,01051
NM_000576	2q14	-3,96	0,01115	-4,17	0,02509	-3,74	0,01328
NM_004744	4q32.1	-2,83	0,02089	-6,37	0,00468	-2,74	0,02371
NM_003914	13q12.3-q13	-3,62	0,01219	-4,80	0,01427	-3,52	0,01345
NM_001248	3p21.	-3,53	0,00307	-4,49	0,00413	-4,47	0,00130
NM_014059	13q14.11	-4,22	0,01758	-4,63	0,03430	-4,09	0,01921
NM_016321	15q25	-3,61	0,00001	-5,15	0,00001	-4,21	0,00000
NM_003866	4q31.21	-3,07	0,00182	-6,57	0,00041	-3,53	0,00100
NM_030666	6p25	-4,59	0,00272	-4,98	0,00649	-3,81	0,00508
ENST00000278919	11q24.2	-4,13	0,00271	-5,50	0,00347	-4,89	0,00153
NM_173080	1q21.3	-2,43	0,04329	-8,01	0,00319	-5,06	0,00342
NM_019618	2q12-q21	-3,46	0,00194	-9,54	0,00029	-6,44	0,00022
NM_004867	Xq13.3-Xq21.2	-7,33	0,00029	-7,19	0,00107	-6,52	0,00040
NM_000396	1q21	-9,90	0,00817	-9,98	0,02173	-5,43	0,02872
NM_018159	Xp11.22	-10,58	0,00006	-7,67	0,00051	-10,74	0,00006
NM_198460	1p22.2	-9,07	0,00756	-15,12	0,00851	-8,20	0,00931
NM_000673	4q23-q24	-15,66	0,00010	-16,41	0,00033	-13,87	0,00013
NM_002083	14q24.1	-25,53	0,00008	-27,97	0,00024	-12,86	0,00028
NM_002277	17q12-q21	-32,94	0,00009	-26,44	0,00047	-29,00	0,00011



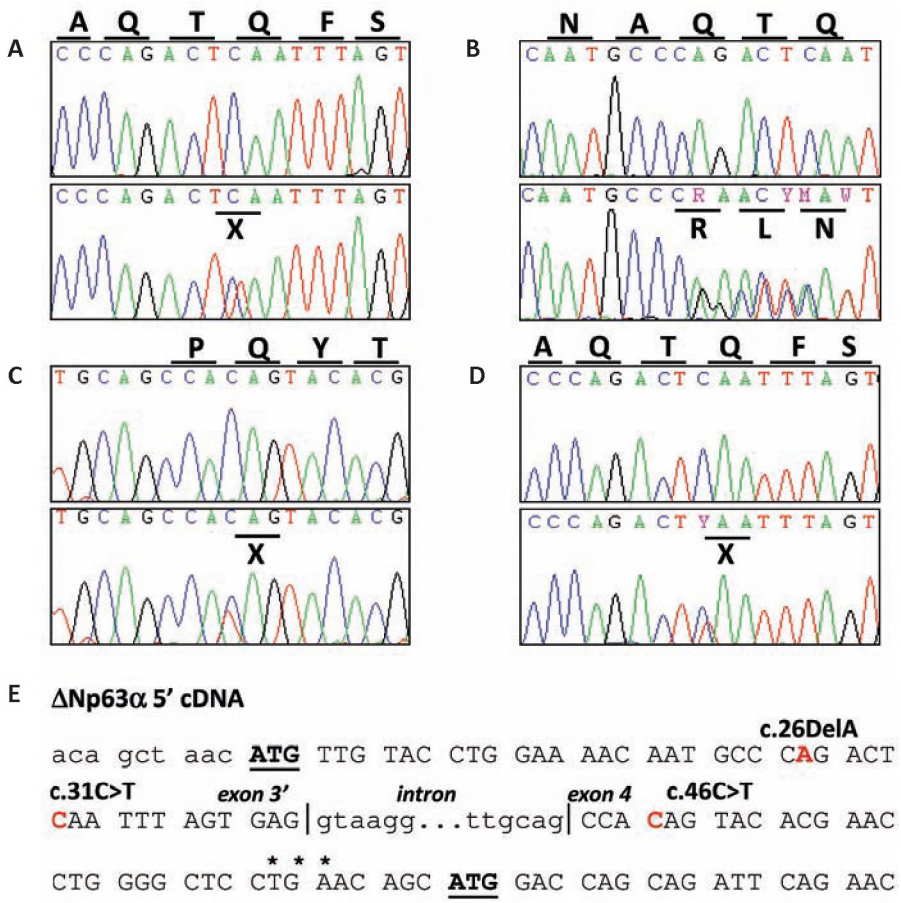
Color Figures



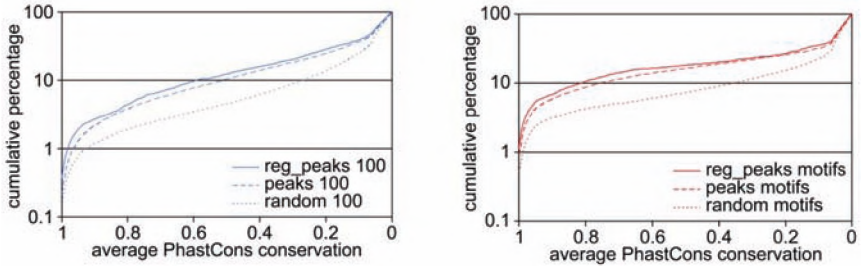
Chapter 4 . Figure 1. Limb and ectodermal phenotype in ADULT syndrome patients with p.R298 mutation. Limb malformations in p.R298 patients can vary much. **A-B**. Deep median cleft and syndactyly in the limbs of the Finnish ADULT syndrome patient (I/1) with p.R298G mutation. **C-D.** Mildly affected limbs in the Italian patient (II/1) with p.R298Q mutation. **E-F.** Severely affected hands and non-affected feet of Danish ADULT patient (II/1) with p.R289Q mutation. **G.** Excessive freckling in Danish patient (I/1). **H.** Hypodontia and loss of permanent teeth in Danish patient (I/1). I. Hypoplastic nipples in Danish patient (II/1).



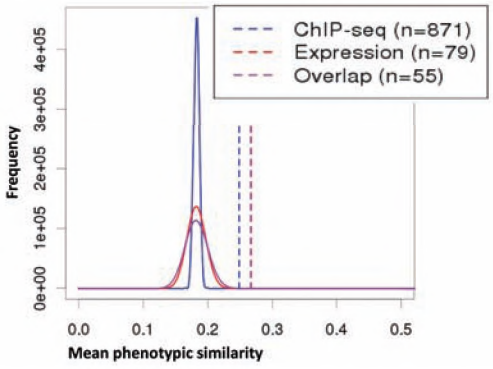
Chapter 4. Figure 3. Undifferentiated oral squamous cell carcinoma (SCC) of the Finnish ADULT syndrome (p.R298G) patient. The SCC oral mucosa (on the left) shows strong p63 immunoreactivity, whereas the p63 expression in the healthy mucosa is strongest in the basal layer and gradually decreased in the suprabasal layers (on the right). (Magnification is 200x.)



Chapter 6. Figure 2. Pathogenic mutations in three RHS/AEC syndrome families. Direct sequencing of genomic DNA from four AEC/RHS patients revealed N-terminal mutations in the p63 gene. **A.** In family 1 a heterozygous nucleotide change c.31C>T was found in affected mother and daughter in exon 3'. The upper chromatogram illustrates a control sequence and the lower is from the affected mother. **B.** In family 3, a heterozygous deletion c.26delA was detected in the index patient in exon 3'. The upper sequence is a control and the lower is from the patient. **C.** In family 2 a heterozygous nucleotide change c.46C>T (ΔNp63-isoform) was detected in exon 4 in the index patient. The upper chromatogram illustrates a control DNA sequence and the lower is from the patient. **D.** Chromatogram of the sequenced cDNA of keratinocytes from the mother of family 1 reveals the same heterozygous nucleotide change detected in the genomic DNA (Fig. 2A). Mutated RNA is present in the cells and is not degraded by the nonsense mediated RNA decay as expected. **E.** The translated sequence of ΔNp63α (AF075431) (capitals) contains two AUG sites (bold, underlined) in the two first exons (exon 3' and 4, | indicates the exon boundary). The mutated nucleotides: c.26A, c.31C and c.46C (indicated red and bold) are located between these two initiation sites. c.26delA mutation leads to a frameshift, which causes a PTC (indicated by ***) only 5 nucleotides upstream of the second AUG site. c.31C>T changes the codon CAA into a termination codon TAA (this PTC is 44 nucleotides upstream from the second AUG). c.46C>T changes codon CAG into a termination codon TAG causing a PTC 29 nucleotides upstream of the second AUG. The second initiation codon is flanked by a strong Kozak sequence, where the most important nucleotides (purine at position -3 and a guanine at position +4) are conserved suggesting its use in translation re-initiation.

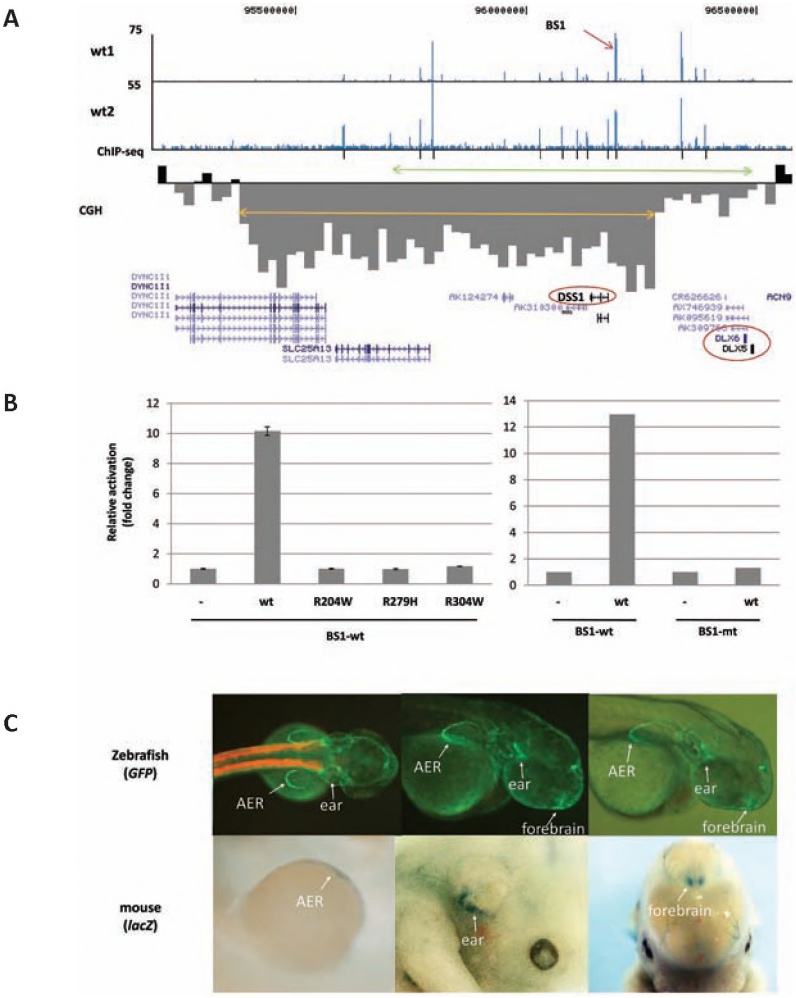


Chapter 7. Figure 3. Conservation of p63 binding sites and motifs in vertebrates. The percentage of p63 binding sites (y-axis) is shown for decreasing PhastCons conservation scores (x-axis). **A.** The average PhastCons conservation score of 100-nucleotide regions centered at the summit of 11,425 p63 binding peaks (peaks 100), of 198 peaks located near 328 differentially regulated genes in three patients (reg_peaks 100), and of 100,000 regions randomly chosen from the whole genome (random 100). **B.** The average PhastCons conservation score of 10nt p63 motifs from 10,675 motifs in the p63 peaks detected in the genome (peaks motifs), from 294 motifs near differentially regulated genes (reg_peaks motifs), and from 7,600 motifs found in random genomic regions (random motifs).

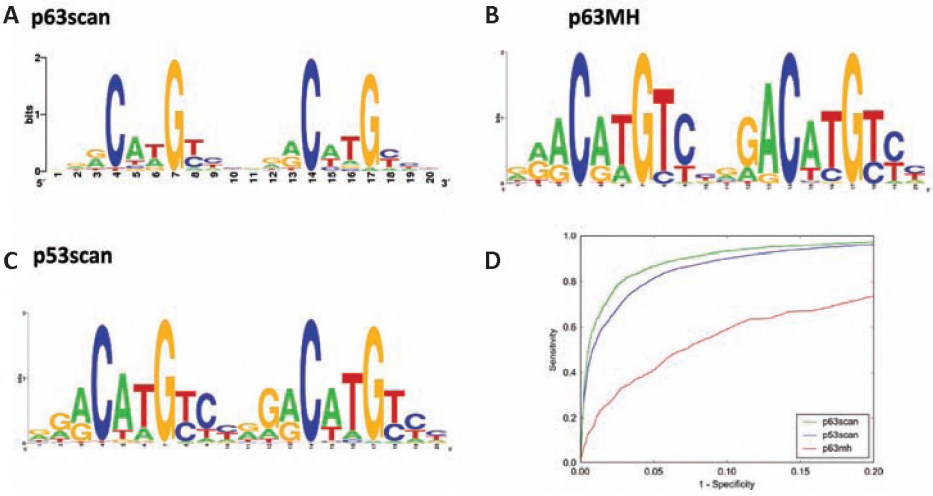


Feature description	P-value
Epithelium	8,34E-11
Adhesion	2,54E-09
Extracellular Matrices	1,32E-08
Basal Lamina	1,38E-07
Membrane	6,28E-07
Corneal Stroma	1,64E-06
Cell, Epithelial	3,19E-06
Dentition	4,26E-06
Extracellular Space	1,14E-05
Hyalin	1,48E-05

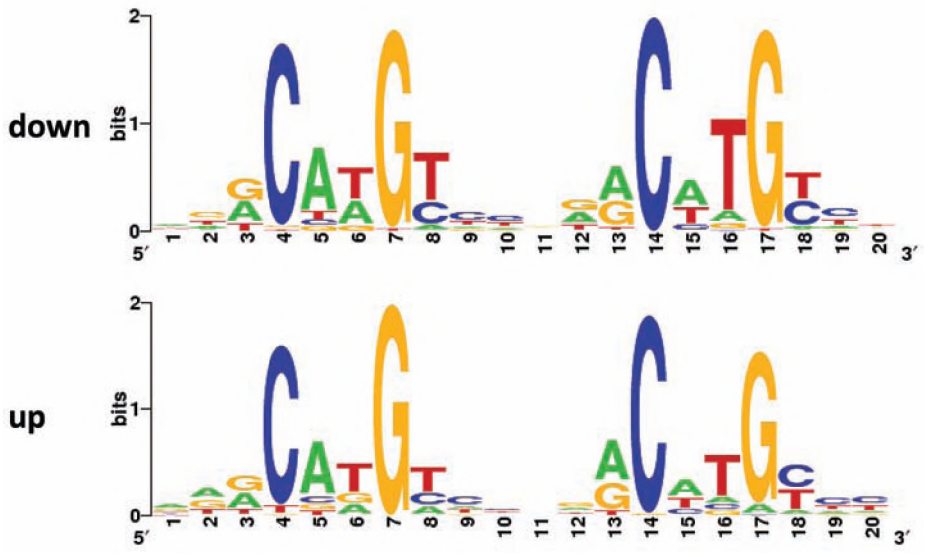
Chapter 7. Figure 5. Association of p63 potential target genes with diseases. A. Genes identified in ChIP-seq and expression analyses and the 198 potential direct target genes (overlap, differentially expressed genes with binding sites) were examined using OMIM disease base to determine their associated diseases (871, 79 and 55 OMIM IDs, respectively) and the affected feature terms in these diseases. The similarity scores from diseases associated with each group of genes are indicated with dashed lines. Diseases associated with genes identified in expression analysis (red) and the potential direct target genes (overlap, purple) showed very similar affected features and are not distinguishable from each other. Diseases linked to genes identified from ChIP-seq analysis (blue) also showed significantly similar features. The same number of diseases associated with each group of genes are randomly selected from the OMIM database. The distribution of the similarity scores from these randomized diseases are calculated and shown in solid lines. **B.** Over-represented feature descriptions in diseases associated with 198 potential target genes.



Chapter 7. Figure 6. Function analysis of p63 binding sites at SHFM1 locus on chromosome 7. A. A screenshot of UCSC genome browser shows the p63 binding profile from two HKC cell lines (wt1 and wt2) and several strong binding sites were identified near *DLX5* and *DLX6* on chromosome 7. Ultra-high Comparative Genomic Hybridization array analysis performed with DNA of a SHFM1 patient showed a chromosomal deletion of 880 kb on chromosome 7 (95,390,000-96,270,000, hg18, the orange arrow) which includes *DSS1*, *SLC25A13* and part of *DYNC1I1* but not *DLX5* and *DLX6*. Breakpoints and 20kb averaged log2 ratios were visualized in the genome browser. A previously reported minimal deletion determined by markers D7S527 and D7S1796 (30) is marked with a green arrow. **B.** The p63 binding site SHFM1-BS1 was tested in transient transfection assays. Transcription of the luciferase reporter gene was strongly activated by **Δ**Np63α through SHFM1-BS1 binding site and the activation was abolished by *p63* EEC mutations p.R204W, p.R279H and p.R304W (left panel). Activation was also abolished when point mutations were introduced into the p63 binding motif in the SHFM1-BS1 binding site (right panel). **C.** SHFM1-BS1 was cloned in reporter constructs carrying the GFP or the LacZ gene to generate transgenic zebrafish and mice, respectively. Expression of the GFP gene in zebrafish and the LacZ gene in mice showed that SHFM1-BS1 can control gene expression at the apical ectodermal ridge (AER), ear and forebrain. The red fluorescence expressed in the muscles in the first zebrafish panel corresponds to the positive control of transgenesis.



Chapter 7. Supplementary figure 2. Comparison of the p63 motif identified in this work with previously reported motifs. A. The p63 motif was identified by de novo motif analysis where 20% of the binding sites were used to predict motifs, and the resulting motifs were scored for significance using the remaining 80% of the binding sites compared to a set of background sequences. Based on a previously developed p53scan algorithm (4) with this newly identified p63 Positional Weight Matrix (PWM), p63scan was developed. **B-C.** The motifs from p63MH (5) and p53scan (4), respectively. **D.** The performance of p63scan using the de novo identified motif shows far superior sensitivity compared to previously reported p63MH without compromising specificity. Compared to p53scan, which was deduced from p53 binding sites, p63scan performs also slightly better on p63 binding sites. The 80% of the binding site sequences not used for motif prediction, as well as a set of background sequences were scanned using the different algorithms. For each individual threshold the fraction of binding sites with a motif (sensitivity) as well as the fraction of background sequences with a motif (1 – specificity) were calculated.



Chapter 7. Supplementary figure 6. Motif analysis of binding sites near up- or down regulated genes. The de novo motif search on binding sites of up- or down-regulated genes revealed no major difference in p63 motifs.